REMARKS/ARGUMENTS

Claims 24-30, 36, 37, 40 and 43-56 are in the case. No amendments are presented with this Response.

At the outset, the undersigned wishes to thank the Examiner (Dr. Winkler) for discussing this case by telephone on April 28, 2005, during which the outstanding action was discussed. It was explained that the references to "activity" in the claims are references to biological activity. This will be clear, for example, from the paragraph bridging pages 37 and 38 of the application.

Referring to the enablement rejection, the Examiner indicated during the telephone interview on April 28, 2005, that she would like to receive a copy of the slide presentation made by Dr. Kenten during the interview conducted on November 12, 2004. A copy is attached, together with a copy of the article by Schneekloth et al., "Chemical Genetic Control of Protein Levels: Selective in Vivo Targeted Degradation", *J. Am. Chem. Soc.*, 126, 3748-3754 (2004), referred to in the slides. The first two slides provide a brief outline, written and pictorially, of the present invention. The remaining slides discuss how the invention is used, and has been used by persons of ordinary skill as referenced by the attached article by Schneekloth et al., which describes a general strategy for the design and synthesis of molecules capable of degrading selected proteins *in vivo* via ubiqutination. The paper reports that "Although this technique has been shown to be effective previously in vitro, this is the first example of synthesized molecules which are capable of inducing the degradation of a targeted protein upon addition to cells." (see, Discussion, Schneekloth et al., page 3752). It is believed that

the content of the attached slide presentation accurately reflects the argument presented during the interview.

Based on this and the *in vitro* data in the present specification, Dr. Kenten expressed the view at the interview that one of ordinary skill would have the expectation that the invention will work *in vivo* and that the method would work in patients. Attached are two further references provided by Dr. Kenten (Zhang et al., "Degradation of Target Protein in living cells by small molecule proteolysis inducer", *Bioorganic & Medicinal Chemistry Letters*, 14 (2004) 645-648, and Zhang et al., "Targeted Degradation of Proteins by Small Molecules: A Novel Tool for Functional Proteomics", *Combinatorial Chemistry and High Throughput Screening*, 2004, 7, 689-697) which further support the general ability of compounds of the invention to work *in vivo* (cells).

It is believed that the lack of enablement rejection has been obviated. Reconsideration and withdrawal of that rejection are respectfully requested.

Favorable action on this application is awaited.

Respectfully submitted,

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Attachments: copy of slides presented during the interview held on November 12, 2004; Schneekloth et al. and two Zhang et al. articles.

Controlling Protein Levels In Eukaryotic Organisms

Application/Control Number:
09/880,149

What is the invention:

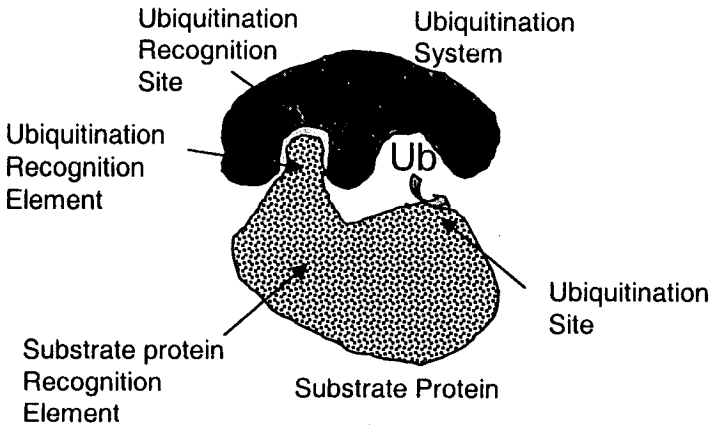
- A) Before: Ubiquitination was known to target proteins for degradation. It was not a general tool to get rid of a specific protein of interest

- B) Realization: We can use ubiquitination as a general tool for controlling levels of any protein.

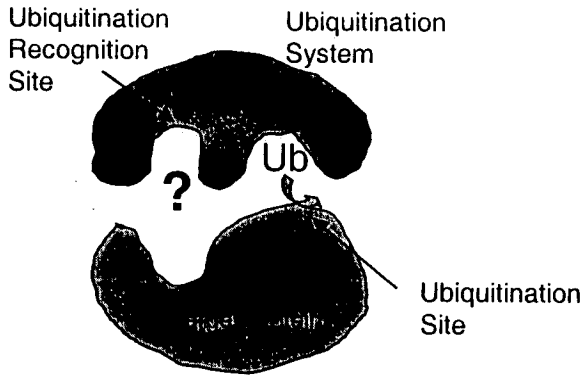
- C) Approach: Use binding reactions to couple target to ubiquitination pathways.

What is the Invention

A) Before

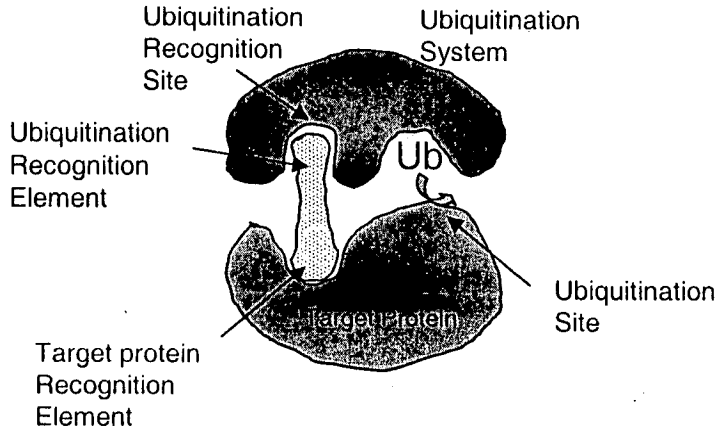


B) Realization



Ub=Ubiquitin

C) Approach



How can the average skilled practitioner use our invention

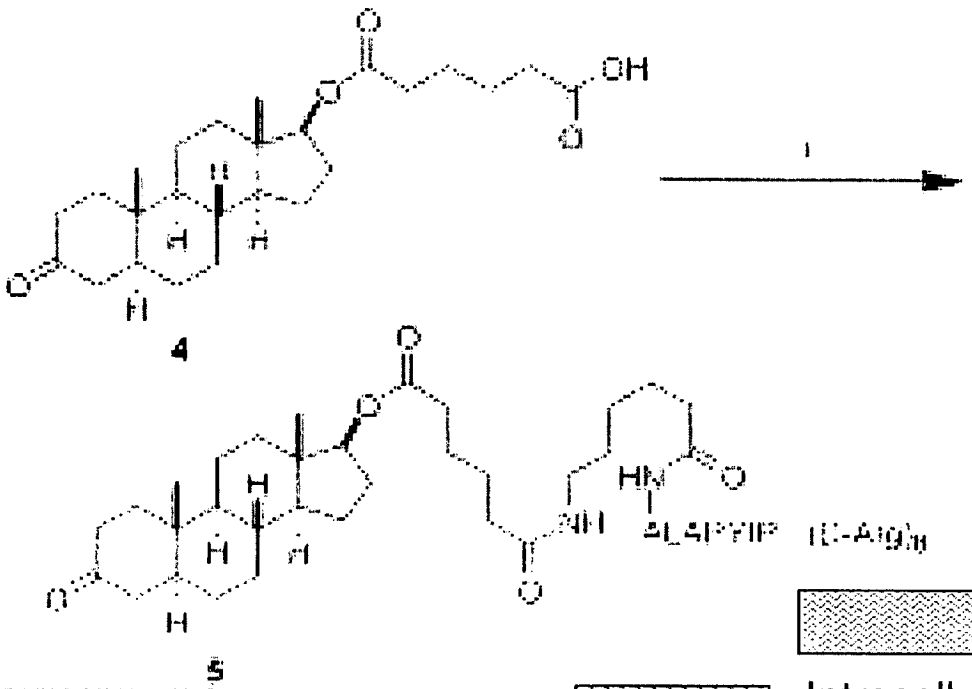
- A) Select protein binding element
  - Selected Examples from application:
    - L-deprenyl, Omeprazole, Clavulanate, chicoric acid, captopril, enalapril, lovastatin, proscar, indinivar, zileuton, L-372,460, apomorphine, N-n-propylnorapomorphine, dihydrexidine, quinpirole, clozapine, haloperidol, nitrocaramiphen, and iodocaramiphen.
- B) Select Ubiquitination recognition element
  - Selected Examples from application:
    - oxidized derivatives of peptides, oxidized amino acid, RXXLGXIXN, Arg-.epsilon.Ahx-Cys, Phe-.epsilon.Ahx-Cys, KX(8-10)DSG(hydrophobic amino acid)XS.
- C) Select Intracellular delivery element (Optional element for improved cell uptake)
  - Selected Examples from application:
    - transportan, homeobox peptides, antennapedia residues 43-58, invasin, galparan.

How has the average skilled practitioner used our invention

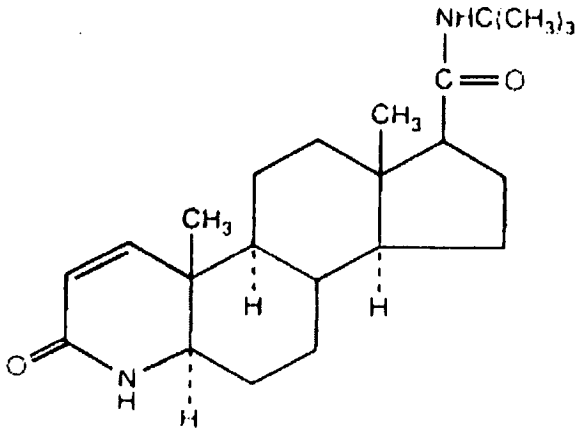
- A) Select protein binding element
  - Selected Steroid:
    - DHT, binding to androgen receptor.
- B) Select Ubiquitination recognition elements
  - Selected peptide:
    - ALAP*YIP, E3 recognition domain, activated by oxidation of proline (P*) to hydroxyproline. Recognized by VBC-Cul2 E3 ubiqitin ligase.
- C) Added Intracellular delivery element
  - Selected peptide:
    - $(D\text{-}Arg)_8CONH_2$

J. Am. Soc. (2004). 126, 3751

Scheme 2. Synthesis of a DHT:HIF1α-Based PROTAC (PROTAC-3)



Proscar from application



Target protein binding element

Application/Control Number: 09/880,149

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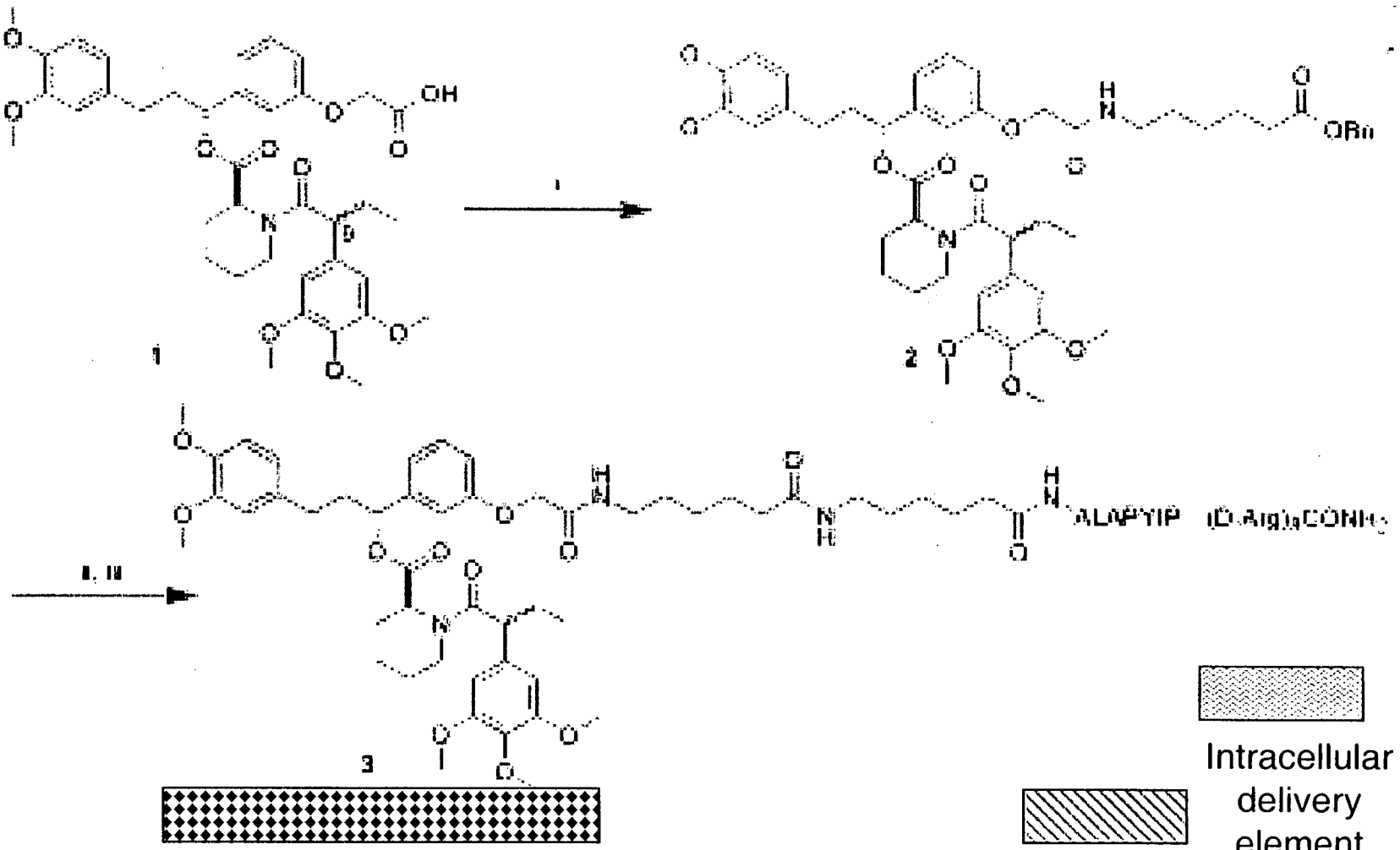
How has the average skilled practitioner used our invention

- A) Select protein binding element
  - Selected derivative of FK506:
    - AP221998, binding to mutant FK506 binding protein (FKBP12).
- B) Select Ubiquitination recognition elements
  - Selected peptide:
    - ALAP*YIP, E3 recognition domain, activated by oxidation of proline (P*) to hydroxyproline. Recognized by VBC-Cul2 E3 ubiqitin ligase.
- C) Added Intracellular delivery element
  - Selected peptide:
    - $(D\text{-}Arg)_8CONH_2$

J. Am. Soc. (2004). 126, 3751

Synthesis of the AP21998/HIF1 α-Based PROTAC



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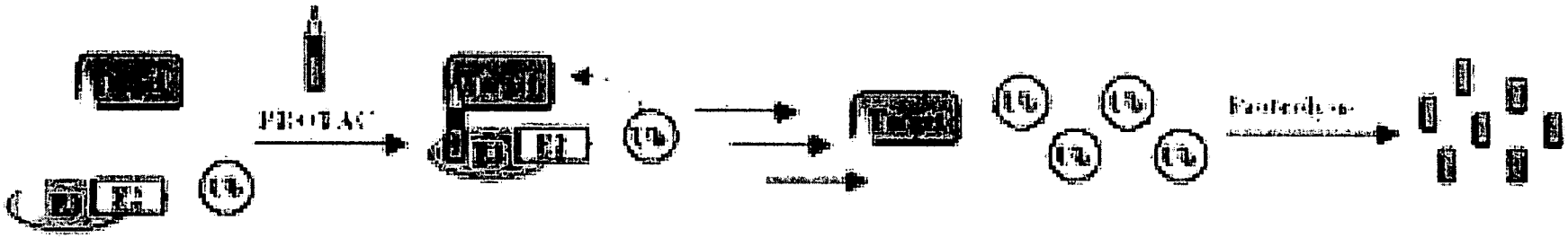
Use of our invention, accomplished



J. Am. Soc. (2004). 126, 3751

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ARTICLES
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Chemical Genetic Control of Protein Levels: Selective in Vivo Targeted Degradation

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Abstract: Genetic loss of function analysis is a powerful method for the study of protein function. However, some cell biological questions are difficult to address using traditional genetic strategies often due to the lack of appropriate genetic model systems. Here, we present a general strategy for the design and syntheses of molecules capable of inducing the degradation of selected proteins in vivo via the ubiquitin−proteasome pathway. Western blot and fluorometric analyses indicated the loss of two different targets: green fluorescent protein (GFP) fused with FK506 binding protein (FKBP12) and GFP fused with the androgen receptor (AR), after treatment with PROteolysis TArgeting Chimeric moleculeS (PROTACS) incorporating a FKBP12 ligand and dihydrotestosterone, respectively. These are the first in vivo examples of direct small molecule-induced recruitment of target proteins to the proteasome for degradation upon addition to cultured cells. Moreover, PROTAC-mediated protein degradation offers a general strategy to create "chemical knockouts," thus opening new possibilities for the control of protein function.

Introduction

The selective loss of critical cellular proteins and subsequent analysis of the resulting phenotypes have proven to be extremely useful in genetic studies of in vivo protein function. In recent years, genetically modified knockout cell lines and animals have allowed biological research to advance with unprecedented speed. Chemical genetic approaches, using small molecules to induce changes in cell phenotype, are complementary to traditional genetics. Many chemical genetic strategies use knowledge gained from natural product mode of action studies,[1−3] while others employ chemical inducers of dimerization to manipulate intracellular processes.[4−7] To date, however, there have been few attempts to design small molecules which induce the destruction (rather than inhibition) of a targeted protein in an otherwise healthy cell. Access to such reagents would provide a chemical genetic alternative to the traditional ways of interfering with protein function, resulting in "chemical knockouts". Importantly, a small molecule capable of inducing this process could do so without any genetic manipulation of the organism, thus allowing one to target proteins that are not readily accessible by traditional genetic means (i.e., genes essential for proliferation and early development).

Protein expression can be described as occurring on three levels: DNA, RNA, and post-translation. Consequently, interference with protein function may be approached from each of these levels. Genetic knockouts disrupt protein function at the DNA level by directly inactivating the gene responsible for a protein product. On the RNA level, removal of a protein of interest may be accomplished by RNA interference (RNAi). RNAi causes the degradation of mRNA within the cell, preventing the synthesis of a protein, and often resulting in a "knockdown" or total knockout of protein levels. Interference with gene products at the post-translational level would involve degradation of the protein after it has been completely expressed. To date, interference with proteins on the post-translation level is the least explored.

In principle, targeted proteolytic degradation could be an effective way to accomplish the removal of a desired gene product at the post-translational level. Given the central role of

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[#] Department of Pharmacology, Yale University.

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the ubiquitin−proteasome pathway in protein degradation within the cell,[8] reagents capable of redirecting the substrate specificity of this pathway would be useful as experimental tools for modulating cellular phenotype and potentially act as drugs for inducing the elimination of disease-promoting proteins. We present here a general strategy for designing molecules capable of inducing the proteolysis of a targeted protein via the ubiquitin−proteasome pathway, as well as the first evidence that such molecules are effective upon addition to living cells.

Protein degradation, like protein synthesis, is an essential part of normal cellular homeostasis. As the major protein degradation pathway, the ATP-dependent ubiquitin−proteasome pathway has been implicated in the regulation of cellular processes as diverse as cell cycle progression,[9] antigen presentation,[10] the inflammatory response,[11] transcription,[12] and signal transduction.[13] The pathway involves two discrete steps: (i) the specific tagging of the protein to be degraded with a polyubiquitin chain and (ii) the subsequent degradation of the tagged substrate by the 26S proteasome, a multicatalytic protease complex. Ubiquitin, a highly conserved 76 amino acid protein,[14] is conjugated to the target protein by a three-part process. First, the C-terminal carboxyl group of ubiquitin is activated by a ubiquitin-activating enzyme (E1). The thioester formed by attachment of ubiquitin to the E1 enzyme is then transferred via a transacylation reaction to an ubiquitin-conjugating enzyme (E2). Finally, ubiquitin is transferred to a lysine (or, less commonly, the amino terminus) of the protein substrate that is specifically bound by an ubiquitin ligase (E3).[15] Successive conjugation of ubiquitin to internal lysines of previously added ubiquitin molecules leads to the formation of polyubiquitin chains.[16] The resulting polyubiquitinated target protein is then recognized by the 26S proteasome, whereupon ubiquitin is cleaved off and the substrate protein threaded into the proteolytic chamber of the proteasome. Importantly, substrate specificity of the ubiquitin−proteasome pathway is conferred by the E3 ligases. Each E3 ligase or recognition subunit of a multiprotein E3 ligase complex binds specifically to a limited number of protein targets sharing a particular destruction sequence. The destruction sequence may require chemical or conformational modification (e.g., phosphorylation) for recognition by E3 enzymes.[17,18]

Recently, we demonstrated a strategy for inducing the ubiquitination and ensuing proteolytic degradation of a targeted protein in vitro. This approach uses heterobifunctional molecules known as PROteolysis TArgeting Chimeric moleculeS (PROTACS), which comprise a ligand for the target protein, a linker moiety, and a ligand for an E3 ubiquitin ligase.[19] In that proof of principle experiment the degradation of a stable protein, methionine aminopeptidase 2 (MetAP-2), was induced in a cellular lysate upon the addition of a PROTAC (referred to as PROTAC-1) consisting of the known MetAP-2 ligand, ovalicin, joined to a peptide ligand for the ubiquitin ligase complex $SCF^{\beta TrCP}$. By bridging MetAP-2 and an E3 ligase, PROTAC-1 initiated the ubiquitination and proteasome-mediated degradation of MetAP-2 (Figure 1). We have also recently shown that an estradiol-based PROTAC (PROTAC-2) could promote the ubiquitination of the human estrogen receptor (hERα) in vitro. Furthermore, a dihydrotestosterone (DHT)-based PROTAC (PROTAC-3), when microinjected into cells, was capable of inducing the degradation of the androgen receptor.[20] Encouraged by our success with PROTACS-1, −2, and −3, we next directed our efforts toward the design of molecules capable of inducing proteolysis simply upon addition to cells. Additionally, the design of new PROTACS takes into account the desire to minimize the amount of molecular biological manipulations necessary to effect degradation to perturb the system as little as possible outside the desired degradation.

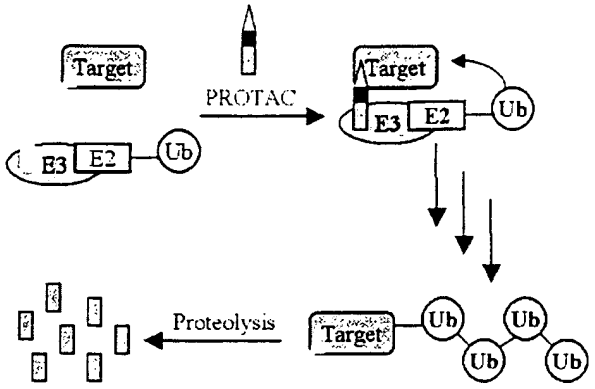

Figure 1. Targeted proteolysis using a PROTAC molecule. Ub = ubiquitin, target = target protein, E3 = E3 ubiquitin ligase complex, and E2 = E2 ubiquitin transfer enzyme.

Results

Development of a Cell Permeable PROTAC: PROTAC-4. For the design of PROTAC-4, we used a protein target/ligand pair developed by ARIAD Pharmaceuticals. The F36V mutation of FK506 binding protein (FKBP12) generates a "hole" into which the artificial ligand AP21998 (**1**) fits via a hydrophobic "bump," thus conferring specificity of this particular ligand to the mutant FKBP over the wild-type protein.[21,22] Inclusion of AP21998 as one domain of PROTAC-4 thus allows it to target (F36V)FKBP12 proteins orthogonally, without disrupting endogenous FKBP12 function. Given the lack of small-molecule E3 ubiquitin ligase ligands, the seven amino acid sequence ALAPYIP was chosen for the E3 recognition domain. This sequence has been shown to be the minimum recognition domain for the von Hippel−Lindau tumor suppressor protein (VHL),[23] part of the VBC−Cul2 E3 ubiquitin ligase complex. Under normoxic conditions, a proline hydroxylase catalyzes the hydroxylation of hypoxia inducible factor 1α (HIF1α) at P564[24]

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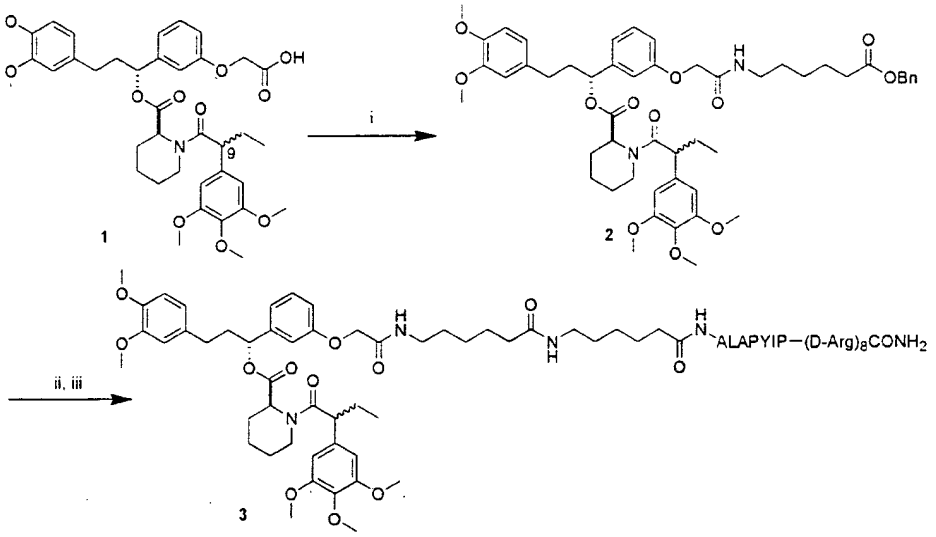
Scheme 1. Synthesis of the AP21998/HIF1 α-Based PROTAC[a]



[a] (i) $H_2N(CH_2)_5CO_2Bn$, EDCI, DMAP. (ii) H_2, Pd/C. (iii) $H_2N(CH_2)_5CONH$-ALAPYIP-(D-Arg)$_8$-NH_2, PyBrOP, DIPEA, DMF.

(the central proline in the ALAPYIP sequence), resulting in recognition and polyubiquitination by VHL. HIF1α is thus constitutively ubiquitinated and degraded under normoxic conditions.[25,26] Finally, a poly-D-arginine tag was included on the carboxy terminus of the peptide sequence to confer cell permeability and resist nonspecific proteolysis. Polyarginine sequences fused to proteins have been shown to facilitate translocation into cells[27,28] via a mechanism that mimics that of the Antennapedia[29] and HIV Tat proteins.[30] Because a molecule fused to the polyarginine sequence should in principle be cell permeable, the necessity of PROTAC microinjection is circumvented. This design element also allows greater flexibility in the types of ligands that could be used in future PROTACs, since polarity of the compound is no longer an issue for membrane permeability. It was hypothesized that PROTAC-4 would enter the cell, be recognized and hydroxylated by a prolyl hydroxylase, and subsequently be bound by both the VHL E3 ligase and the mutant FKBP12 target protein. PROTAC-mediated recruitment of FKBP12 to the VBC−Cul2 E3 ligase complex would be predicted to induce FKBP12 ubiquitination and degradation as in Figure 1.

The F36V FKBP12 ligand AP21998 (**1**) was synthesized as previously described,[21,22] as an approximately 1:1 mixture of diastereomers at C9. Treatment of **1** with the benzyl ester of aminocaproic acid followed by removal of the benzyl group afforded **2** in 85% crude yield after two steps. It is important to note that although this material was carried through as a mixture of two diastereomers at C9, each diastereomer has previously been shown to bind to the target.[22] Standard peptide coupling conditions were used to label the peptide sequence. HPLC purification yielded **3** (PROTAC-4) with 17% recovery from **1** (Scheme 1).

To monitor the abundance of the targeted protein, a vector capable of expressing the mutant FKBP12 fused to enhanced green fluorescent protein (EGFP) was generated. In this way, proteolysis of FKBP12 could be monitored by loss of intracellular fluorescence. This vector was then used to generate a HeLa cell line stably expressing the EGFP−(F36V)FKBP12. Bright field and fluorescent photographs of the cells were taken before and 2.5 h after treatment with PROTAC-4 (**3**). As shown in Figure 2A−D, EGFP−FKBP12 was retained in those cells treated with DMSO, but lost in cells treated with 25 μM PROTAC-4 for 2.5 h. Western blot analysis of cells treated with PROTAC-4 also indicated loss of EGFP−FKBP12 relative to an equal number of cells treated with DMSO (Figure 2I). As a control, cells were treated with uncoupled **1** and the HIF-polyarginine peptide fragment (Figure 2E,F). These cells retained fluorescence, indicating that the two domains require a chemical bond to each other to exert a biological effect. To investigate whether VHL was required for PROTAC-4-mediated EGFP−FKBP12 degradation, the renal carcinoma cell line 786-O[31] was used. 786-O cells failed to produce VHL protein and thus lack a functional VBC−Cul2 E3 ligase complex. 786-O cells stably expressing the degradation substrate EGFP−FKBP12 retained fluorescence despite treatment with 25 μM PROTAC-4 for 2.5 h (Figure 2G,H), confirming that the E3 ligase is required for PROTAC-4 activity. Finally, similar cell density and morphology in bright field images before (Figure 2I) and after (Figure 2J) treatment with 25 μM PROTAC-4 for

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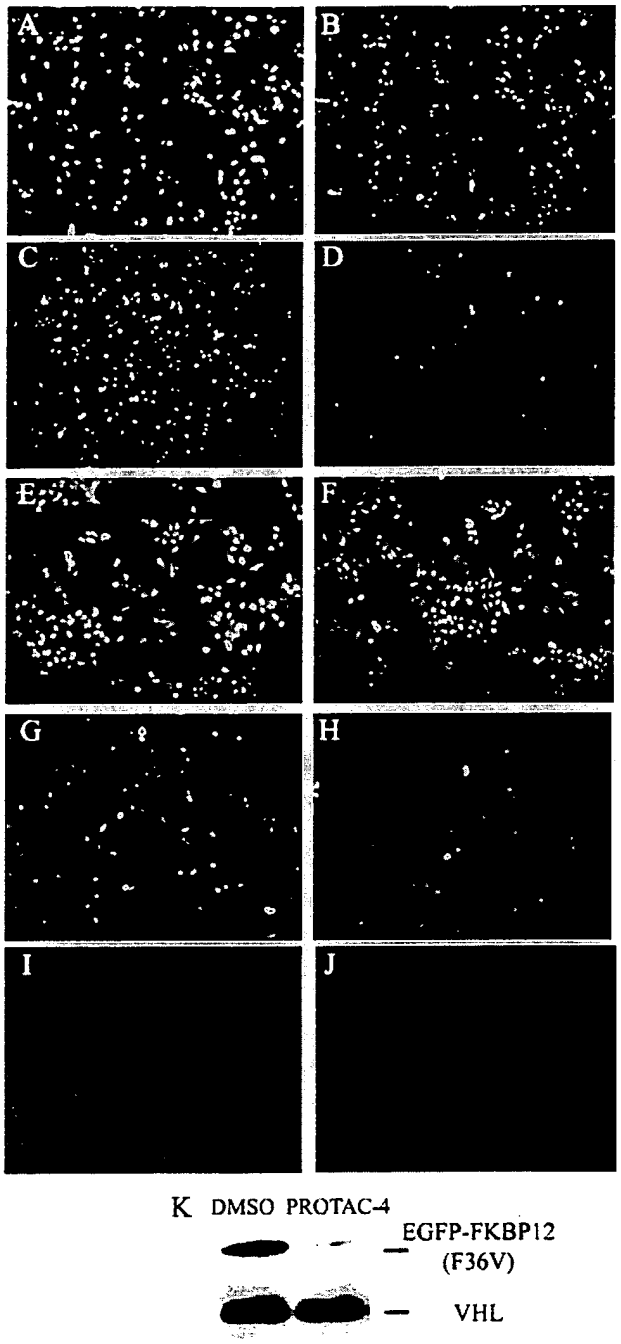
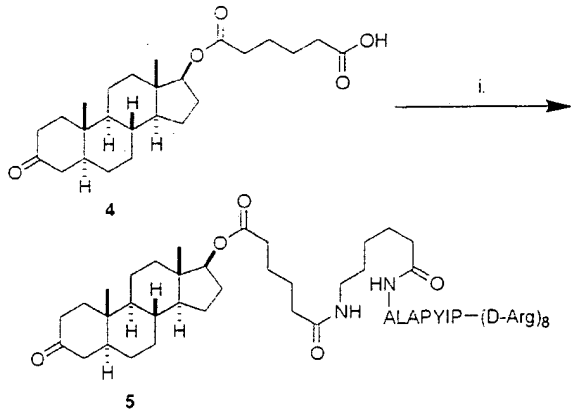


Figure 2. PROTAC-4 (3) mediates EGFP−FKBP degradation in a VHL-dependent manner. No change in fluorescence is observed before (A) and 2.5 h after (B) treatment in DMSO control, while a significant change is observed between before (C) and 2.5 h after (D) treatment with 25 μM 3. Cells treated with 25 μM 1 and 25 μM HIF-(D-Arg)$_8$ peptide show no difference before (E) and 2.5 h after (F) treatment. 786-O$^{EGFP-FKBP}$ cells do not lose fluorescence before (G) or 2.5 h after (H) treatment with 25 μM 3. Bright field images of cells before (I) and 2.5 h after (J) treatment with 25 μM 1 affirm constant cell density and morphology. Western blot analysis (K) with monoclonal anti-GFP antibodies confirms loss of EGFP−FKBP in cells treated with 25 μM 3 (PROTAC-4) for 2.5 h compared to an equal load from vehicle (DMSO) treated cells.

2.5 h confirm that cells are capable of surviving treatment with a PROTAC molecule.

Implementation of a DHT-Based PROTAC: PROTAC-5. To test the robustness of this approach for the induction of

Scheme 2. Synthesis of a DHT/HIF1 α-Based PROTAC (PROTAC-3)[a]



[a] (i) $H_2N(CH_2)_5CONH$-ALAPYIP-(D-Arg)$_8$-NH_2, EDCI, DMAP, DMF.

intracellular protein degradation, we next used a well understood protein−ligand pair which occurs in nature. The testosterone/androgen receptor pair was particularly attractive because it has been shown that the androgen receptor (AR) can promote the growth of prostate tumor cells, even in some androgen-independent cell lines.[32] In those same cell lines, it has been shown that inhibition of AR represses growth.[32] We hypothesized that a PROTAC could be utilized to degrade AR, potentially yielding a novel strategy to repress tumor growth. With this in mind, the design of PROTAC-5, **5**, contains DHT as the ligand for AR as well as the HIF-polyarginine peptide sequence which was successful with PROTAC-4. Known DHT derivative **4**[33] was successfully coupled to the HIF-polyarginine peptide with standard peptide coupling conditions (Scheme 2). To monitor protein degradation by fluorescence analysis, HEK293 cells stably expressing GFP-AR (293^{GFP-AR}) were treated with increasing concentrations of PROTAC-5. Within 1 h, a significant decrease in GFP-AR signal was observed in cells treated with 100, 50, and 25 μM PROTAC-5, but not in the DMSO control (Figure 3, parts A−F, I, L). Western blot analysis with anti-AR antisera verified the downregulation of GFP-AR in cells treated with 25 μM PROTAC-5 compared to DMSO control or nontreated cells (Figure 3M). PROTAC-5 concentrations lower than 25 μM did not result in GFP-AR degradation (data not shown). Pretreatment of cells with epoxomicin, a specific proteasome inhibitor,[34] prevented degradation of GFP-AR (Figure 3, part H: light field, K: fluorescent), indicating that the observed degradation was proteasome-dependent. This result was also verified by Western blot (Figure 3N). It should be noted that decreased cell density in the epoxomicin experiments are most likely due to the inherent toxicity of epoxomicin itself, rather than from a toxic effect of the PROTAC. This is supported by the viability of cells treated with PROTAC-5, as seen in Figure 3B,C.

Competition experiments with testosterone also inhibited PROTAC-5 from inducing GFP-AR degradation (Figure 4 A-D). In addition, cells treated only with testosterone retained all fluorescence, as did cells treated with the HIF-polyarginine peptide (Figure 4G,H). Finally, cells treated with both testoster-

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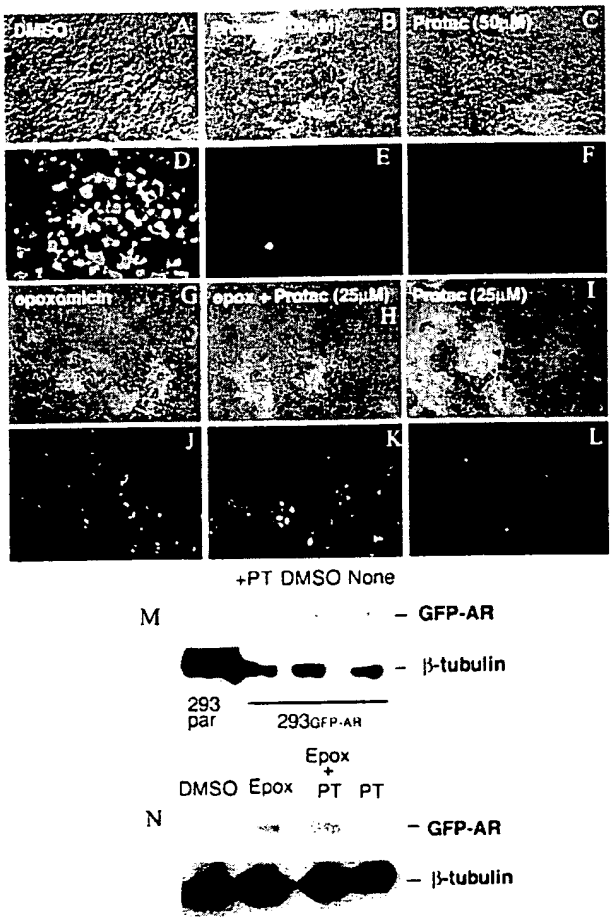

Figure 3. DHT-HIF PROTAC-5 (**5**) mediates GFP-AR degradation in a proteasome-dependent manner. One hour after treatment, 293^{GFP-AR} cells treated with a 100 μM (B light field, E fluorescent) or 50 μM (C light field, F fluorescent) concentration of **5** lose fluorescence, while the DMSO control (A light field, D fluorescent) retains fluorescence. Cells treated with 10 μM epoxomicin (G light field, J fluorescent) and pretreated with 10 μM epoxomicin for 4 h followed by treatment with 25 μM **5** for 1 h (H light field, K fluorescent) retain fluorescence, while cells treated only with 25 μM **5** lose fluorescence after 1 h (I light field, L fluorescent). Western blot analysis confirms loss of GFP-AR after treatment with PROTAC **5** (+PT) relative to a loading control (M), while inhibition of the proteasome with epoxomicin (Epox) inhibits degradation (N).

one and the HIF-polyarginine peptide together also retained fluorescence, indicating again that both domains needed to be chemically linked to observe degradation (Figure 4F). It is important to note again that the cells survived treatment with PROTAC-5, indicating that the strategy of utilizing the ubiquitin–proteasome pathway for targeted degradation does not necessarily cause a toxic effect.

Discussion

These experiments highlight the general applicability of a novel strategy to target and degrade proteins in vivo. Although this technique has been shown to be effective previously in vitro, this is the first example of synthesized molecules which are capable of inducing the degradation of a targeted protein upon addition to cells. Use of a GFP fusion protein provided a convenient method to monitor PROTAC-induced degradation, but is not inherently necessary to the design of the molecule. In principle, no molecular biological manipulations are needed to implement a PROTAC molecule. This technique therefore

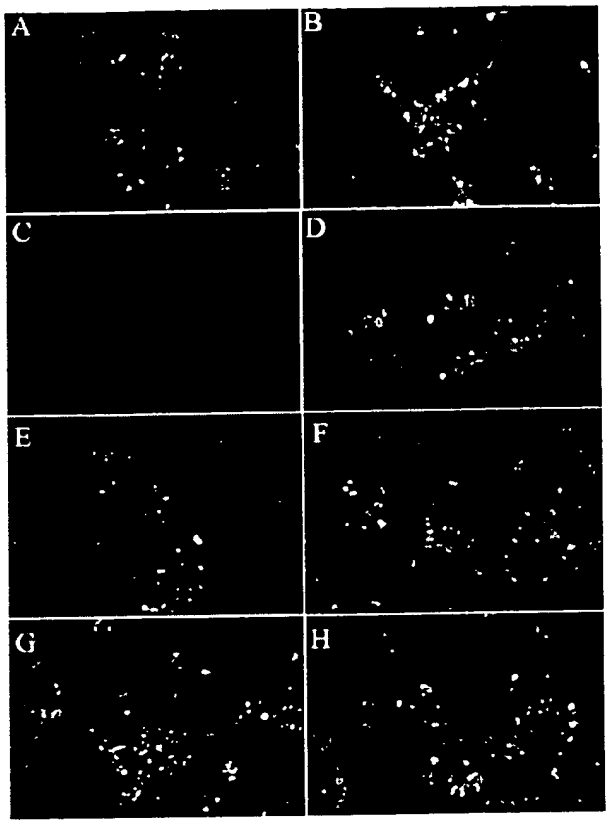

Figure 4. A chemical bond between the HIF-(D-Arg)$_8$ peptide and DHT is required for PROTAC-5-induced degradation of GFP-AR. Cells were treated with (A) no treatment, (B) DMSO (equal volume), (C) 25 μM PROTAC-3, (D) 25 μM PROTAC-5 + 10-fold molar excess testosterone, (E) 25 μM PROTAC-5 + 10-fold molar excess (250 μM) HIF-D-Arg peptide, (F) 25 μM HIF-D-Arg peptide + 25 μM testosterone added separately, (G) 25 μM DHT, and (H) 25 μM HIF-D-Arg peptide.

provides a novel approach to the study of protein function without genetically modifying the host cell. Moreover, the modularity of the PROTAC design offers the possibility to synthesize similar PROTAC molecules targeting a variety of intracellular targets. These experiments have shown that the ligand for the target protein can be varied using both natural and synthetic ligands to degrade effectively targeted GFP fusion proteins. Although the linker length has not been fully explored, a spacer consisting of two aminocaproic acids (12 atoms) has been shown to be flexible enough to accommodate some structural variation in the target and E3 ligase proteins yet remain functional. Since ubiquitination occurs most commonly on an exposed lysine, different spacer lengths may be required to accommodate the structures of different target proteins.

Small molecules have previously been implicated in inducing ubiquitination and degradation of proteins; most notably geldanamycin derivatives act by controlling target interaction with molecular chaperones.[35–38] However, there are often specificity issues with these approaches, and the exact mechanism of

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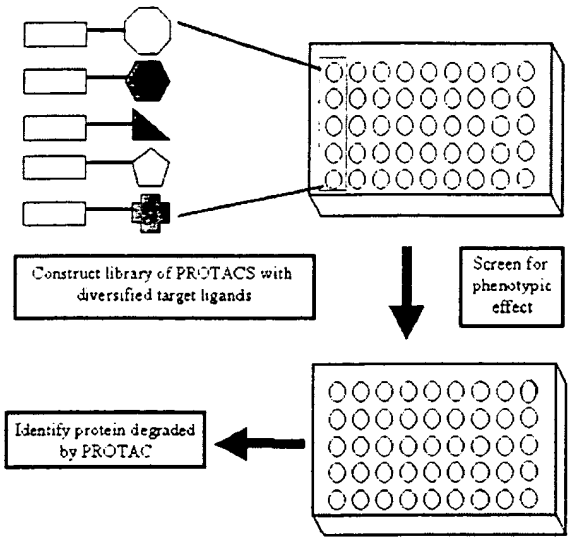

Figure 5. Potential use of PROTACS in a chemical genetic screen.

induced degradation is not clear. Interference with gene products at the post-translational level has also been successfully demonstrated by Howley and co-workers,[39] who used known protein−protein interacting domains. Their approach, while successful, required significant manipulation of the cell lines in question to observe an effect. Both of these methods are significantly less direct and flexible than PROTACS. In addition, the PROTAC strategy represents the first attempt to develop a general method for small molecule-induced targeted proteolysis via the ubiquitin−proteasome pathway in intact cells.

PROTACS could in principle be used to target almost any protein within a cell and selectively initiate its degradation, resulting in a "chemical knockout" of protein function. A notable advantage to this strategy is that proteolysis is not dependent on the active-site inhibition of the target; any unique site of a protein may be targeted, provided that there are exposed lysines within proximity for the attachment of ubiquitin. Because some E3 ligases are expressed in a tissue-specific manner, this also raises the possibility that PROTACS could be used as tissue-specific drugs.

Several other applications for this technology can be envisioned. First, PROTACS could be used to control a desired cellular phenotype, for example, via the induced degradation of a crucial regulatory transcription factor which is difficult to target pharmaceutically. "Chemical knockout" of a protein could prove viable as an alternative for a genetic knockout, which would be extremely valuable in the study of protein function. This strategy could also provide significantly more temporal or dosing control than gene inactivation at the DNA or RNA level. Second, libraries of PROTACS could be used to screen for phenotypic effects in a chemical genetic fashion. This strategy could be used either to identify novel ligands for a target or to identify new therapeutically vulnerable protein targets by studying phenotypic change as a result of selective protein degradation (Figure 5). This chemical genetic strategy would employ a library of PROTAC molecules with identical E3 ubiquitin ligase domains but chemically diverse target ligands. After PROTAC library incubation with cultured cells and detection of the desired cellular phenotype (e.g., inhibition of pro-inflammatory signaling), one could identify the protein that was degraded by incubation with the PROTAC. A number of approaches could be used to identify the PROTAC-targeted protein, including affinity chromatography and differential proteomic technologies such as ICAT.[40] In a modification of this strategy, a library of PROTACS could be screened to identify a ligand for a particular target by monitoring degradation of the target protein (e.g., loss of GFP fusion protein). Finally, PROTACS could be used as drugs to remove toxic or disease-causing proteins. This strategy is particularly appealing since many diseases, including several cancers, are dependent on the presence or overexpression of a small number of proteins. The large number of potential uses for this technology, coupled with the success of these experiments, suggests that PROTACS could find broad use in the fields of cell biology, biochemistry, and potentially medicine.

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Experimental Section

A. Materials. (F36V)FKBP12 expression vector was generously provided by ARIAD Pharmaceuticals (Cambridge, MA), and GFP-AR expression plasmid was a gift from Dr. Charles Sawyers (HHMI, UCLA). Epoxomicin[41] and AP21998[21,22] were synthesized as previously described. Dihydrotestosterone and testosterone were obtained from Sigma-Aldrich (St. Louis, MO). Monoclonal antibody recognizing VHL was purchased from Oncogene (San Diego, CA), antibodies recognizing GFP and β-tubulin were obtained from Santa Cruz Biotech (Santa Cruz, CA), and polyclonal antibody against the androgen receptor was from United Biomedical, Inc. (Hauppauge, NY). HEK293, 786-O, and HeLa cells were purchased from the American Type Culture Collection (Manassas, VA). Tissue culture medium and reagents were obtained from GIBCO-Invitrogen (Carlsbad, CA).

B. Tissue Culture. HeLa cells, 786-O cells, and HEK 293 cells were separately cultured in D-MEM supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 mg/mL streptomycin, and 2 mM L-glutamine. All cell lines were maintained at a temperature of 37 °C in a humidified atmosphere of 5% CO_2. To generate cells stably expressing a particular fluorescent target protein, the parent cell line was grown to 70% confluency and transfected using calcium phosphate precipitation of the designated cDNA. Following transfection, cells were split 1:10 into culture medium supplemented with 600 μg/mL G418 (GIBCO-Invitrogen). Individual clones which optimally expressed fluorescent target protein were identified and expanded under selection for further experimentation.

C. Detection of PROTAC-Induced Degradation by Fluorescence Microscopy. Cells stably expressing fluorescent target protein were plated into 96 well plates ($HeLa^{EGFP-FKBP}$ cells plated at 4000 cells/well and $HEK293^{GFP-AR}$ cells plated at 60 000−100 000 cells/well). Synthesized PROTACS were dissolved in DMSO vehicle at a final concentration of 1%. Disappearance of target protein in vivo was monitored by fluorescence microscopy at an excitation wavelength of 488 nm.

D. Detection of PROTAC-Induced Degradation by Western Blot. Whole cell lysates were prepared from HeLaEGFP−FKBP cells treated with PROTAC-4 and with HEK293GFP-AR cells treated with PRTOAC-5 by lysing the cells in hot Laemmli buffer. Lysates were subjected to 8% polyacrylamide gel electrophoresis, and the proteins were transferred to nitrocellulose membrane. Membranes were blocked in 3% nonfat milk in TBS supplemented with 0.1% Triton X-100 and 0.02% sodium azide. Lysates from $HeLa^{EGFP-FKBP}$ cells treated with

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PROTAC-4 were probed with anti-GFP (1:1000) and anti-VHL (1:1000) antibodies, and HEK293GFP-AR cells treated with PROTAC-5 were probed with anti-androgen receptor (1:1000) and anti-β-tubulin (1:200) antibodies. Blots were developed using chemiluminescent detection.

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Supporting Information Available: Preparation and characterization information for compounds **3** and **5** and the HIF-polyarginine peptide (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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Degradation of target protein in living cells by small-molecule proteolysis inducer

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Abstract—Ubiquitin-dependent proteolysis of cellular proteins is one of the major pathways to regulate protein function post-translationally. Here we demonstrate a potentially general method of degrading any targeted proteins by the ubiquitin-dependent proteolysis in living cells, using small-molecule proteolysis inducer (SMPI).
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While the use of small molecules as therapeutic agents or lead compounds in drug development process has been a core of the modern pharmaceutical industry, these cell-permeable compounds have been increasingly used as molecular probes to explore intracellular processes.[1–3] Consequently, development of small-molecule modulators of proteins has become a major task to explore protein function, particularly, in the postgenomic era. Currently, most efforts in identifying small-molecule modulators of proteins have been pharmacologically driven. Typically, these modulators control signaling events by directly binding to intracellular targets, either repressing or stimulating biological processes. Meanwhile, in living cells many important biological processes are regulated by the ubiquitin-proteasome pathway,[4–6] which is one of the major pathways to regulate protein function post-translationally.

The ubiquitin-proteasome pathway is the principle conduit for protein turnover in all eukaryotic cells. Ubiquitin-dependent proteolysis involves the assembly of a ubiquitin chain on a substrate, which targets the attached protein for degradation by the 26S proteasome.[7–9] Ubiquitin is first activated at its C-terminus by adenylation and formation of a thioester bond with the ubiquitin-activating enzyme, E1. Activated ubiquitin is subsequently transferred from E1 to a cysteine residue of a member of the family of ubiquitin-conjugating (E2) enzymes. Finally, ubiquitin is transferred from E2 enzyme to a lysine residue of a target protein, either directly or with the assistance of a ubiquitin ligase (E3). Although E2s can directly transfer ubiquitin to model substrates in vitro, most physiological ubiquitination reactions are thought to require the participation of an E3. E3s appear to be the primary source of substrate specificity in the ubiquitination cascade, as they have been shown to bind directly and specifically to substrates.[7–9] Targeting a substrate by an E3 appears to be precisely controlled by posttranslational modifications such as phosphorylation or hydroxylation, since the ubiquitin-proteasome pathway seems to be constitutively active regardless of the status of signaling cascades (i.e., on or off). Consecutive cycles of ubiquitin transfer by E2 to substrate result in the assembly of a multiubiquitin chain on the substrate, which targets it for destruction by the 26S proteasome. Previously, it has been shown that SCF (Skp1-Cullin-F-Box) complex, one of the most-studied E3 ubiquitin ligase complexes,[7–9] can be used to target a protein for ubiquitination and degradation in vitro,[10,11] using a chimeric molecule that recruits a target protein to the SCF complex. However, the approach has not been accomplished in vivo (or in living cells) due in large part to the poor membrane permeability and bioavailability of the chimerae. In addition, when the SCF recognition motif is inserted into a cellular protein, it is shown to direct the degradation of otherwise stable cellular protein both in yeast and in mammalian cells.[12]

Herein, we report for the first time the development of a cell-permeable small-molecule proteolysis inducer

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(SMPI) that exploits the E3 ubiquitin ligase pVHL (von Hippel-Lindau tumor suppressor): hypoxia-inducible factor-1α (HIF-1α) interaction,[13–16] thereby inducing ubiquitination and degradation of a target protein in living cells (Fig. 1). One domain of the SMPI contains the HIF-1α protein-derived octapeptide motif that is recognized by the pVHL E3 ubiquitin ligase complex, whereas the other domain is composed of a protein ligand. In addition, we report for the first time herein that a reversible ligand that non-covalently binds to a target protein can be used for the SMPI approach.

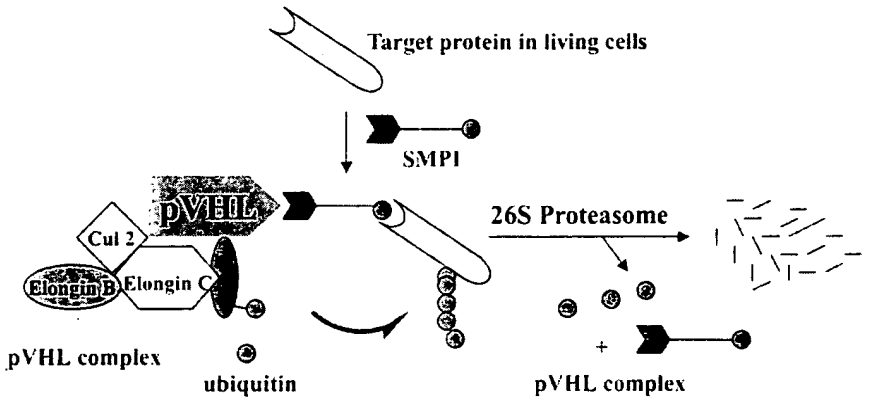

Figure 1. A cell-permeable small-molecule proteolysis inducer (SMPI) recruits target protein to the pVHL complex for ubiquitination and degradation in living cells.

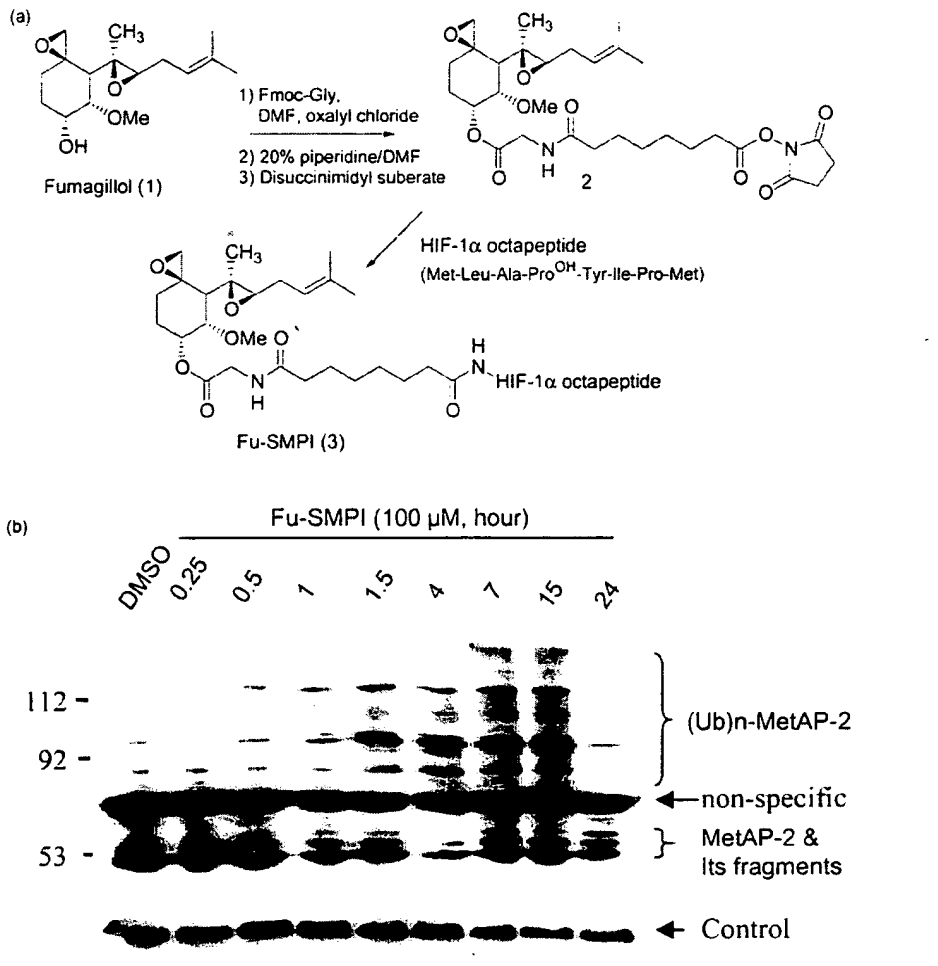

Figure 2. (a) Synthesis of fumagillol-coupled small-molecule proteolysis inducer (Fu-SMPI); (b) Western blots of lysates from Fu-SMPI-treated cells (A549) probed with anti-MetAP-2 antibody: a time-dependent accumulation of ubiquitinated MetAP-2 in lung cancer cells (A549). It should be noted that MetAP-2 is continuously synthesized in living cells throughout the incubation period, whereas all of Fu-SMPI were consumed or decomposed within 24 h, thereby observing no ubiquitinated MetAP-2 after 24 h.

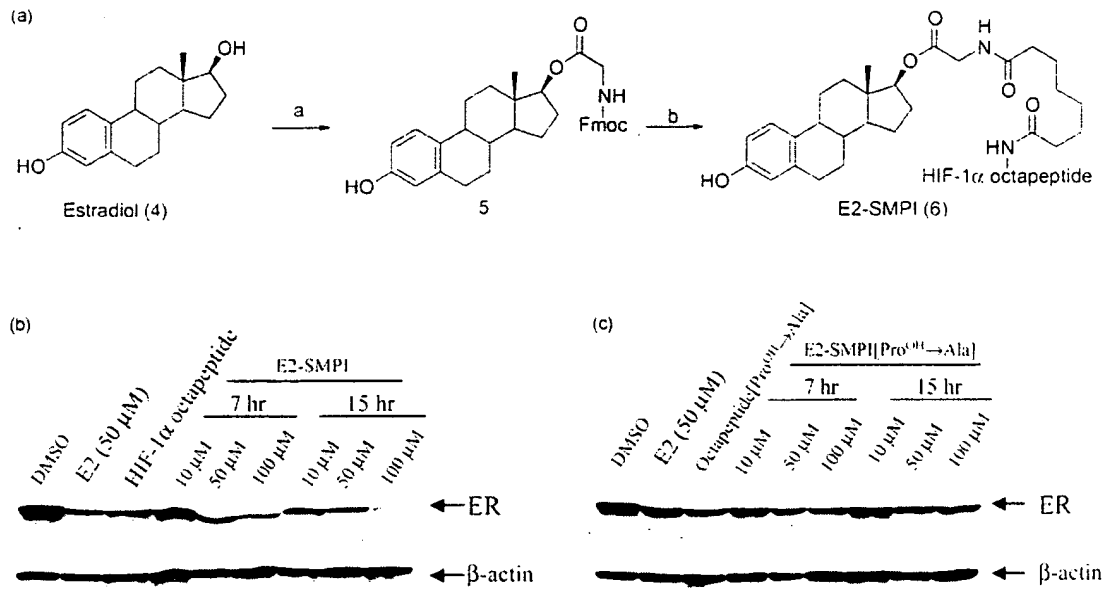

Figure 3. (a) Synthesis scheme of E2-SMPI: (a) (i) Fmoc-Gly-OH, oxalyl chloride/DMF; (ii) E2/DMAP; (b) (i) 20% Piperidine/DMF; (ii) Disuccinimidyl suberate (DSS); (iii) HIF-1α octapeptide or HIF-1α octapeptide[Pro^{OH}—Ala]. Western blots of lysates from E2-SMPI-treated MCF-7 cells probed with anti-ER antibody; (b) E2-SMPI selectively induced ER degradation, where MCF-7 breast cancer cells were treated with 10–100 μM of E2-SMPI, and incubated for 7 h or 15 h before cell lysis and immunoblotting; (c) MCF-7 cells were treated with 10–100 μM of E2-SMPI [Pro^{OH}—Ala], and incubated for 7 h or 15 h. Cells then were lysed, and immunoblotted with anti-ER antibody. All controls were incubated for 15 h.

The HIF-1α plays a key role in the adaptation of mammalian cells during low-oxygen stress,[17] and is rapidly degraded under normal oxygen concentration (or normoxic) conditions by the ubiquitin-proteasome pathway. Degradation of HIF-1α under normoxic conditions is triggered by the hydroxylation of a conserved proline residue (Pro^{564}) that is subsequently recognized by the pVHL E3 ligase, a component of ubiquitin ligase complex that mediates ubiquitination and degradation of HIF-1α.[13,14] Interestingly, it has been shown that a synthetic octapeptide derived from HIF-1α residues 561 to 568, which contains a hydroxyproline at position 564, is sufficient to interact with the pVHL.[13] Taken together, we envision that a chimeric molecule, which is composed of the synthetic HIF-1α octapeptide and a ligand of a target protein, will recruit the target protein to the E3 ligase pVHL for ubiquitination and subsequent degradation by the 26S proteasome (Fig. 1). To this end, we prepared a small-molecule proteolysis inducer, which is designed to target methionine aminopeptidase-2 (MetAP-2), by coupling fumagillol to the HIF-1α octapeptide to yield fumagillol-coupled HIF-1α octapeptide (Fu-SMPI) (Fig. 2a). Fumagillol is an active derivative of the angiogenesis inhibitor fumagillin, which selectively binds to methionine aminopeptidase-2 (MetAP-2)[18,19] that is not known to be ubiquitinated.

When lung cancer cells (A549) were treated with Fu-SMPI, ubiquitinated MetAP-2s were time-dependently accumulated, and eventually degraded (Fig. 2b).[20] Moreover, these high molecular species (multi-ubiquitinated MetAP-2s) were consistently competed away by excess fumagillol (data not shown), confirming that MetAP-2 was selectively ubiquitinated by Fu-SMPI. Given that the covalent nature of fumagillol–MetAP-2 interaction,[10] we next asked whether a reversible ligand-based SMPI induces ubiquitination and degradation of a target protein. To test this, we chose the estrogen receptor (ER) ligand estradiol (E2) and prepared E2-based ER-targeting SMPI in which a HIF-1α octapeptide was linked to estradiol using a synthetic approach similar to that of Fu-SMPI (Fig. 3a). When MCF-7 breast cancer cells were treated with E2-SMPI for 15 h, remarkably, the ER protein was completely disappeared (Fig. 3b). That degradation of the ER was mediated by the pVHL–E2-SMPI interaction was further confirmed by the treatment of E2-SMPI [Pro^{OH}—Ala], which contains a mutant HIF-1α octapeptide and therefore does not interact with the pVHL. In deed, E2-SMPI[Pro^{OH}→Ala] did not induce degradation of the ER as shown in Fig. 3c.

Taken together, these results indicate that the pVHL-mediated SMPI approach can be applied to target proteins that promote diseases. In this study, we developed a cell-permeable small-molecule proteolysis inducer that recruits a target protein for ubiquitination and degradation. Unlike conventional small-molecule ligands that must inhibit target proteins to be useful as therapeutic agents, the advantage of SMPI approach is that any protein ligands including non-functional ligands will be useful in the design of SMPI. In other words, as long as a protein ligand has the ability to interact with a target protein (i.e., either inhibitory, non-functional, or stimulatory interactions), the ligand-based SMPI is expected to ubiquitinate and degrade the target protein, given that SMPI approach is driven solely by the protein-binding ability of ligands but not by the pharmacological activity.

In summary, we report here the development of cell-permeable small-molecule proteolysis inducers (SMPIs)

that exploit the unique characteristics of the E3 ubiquitin ligase (pVHL)-mediated protein degradation pathways. The cell-permeable SMPIs selectively induced ubiquitination and degradation of target proteins in living cells. These results provide the generality of the SMPI approach to target other proteins for the ubiquitin-proteasome pathway, providing the general strategy to degrade proteins that play an important role in disease development and progression and are not considered to be readily 'drugable target'. The potential application of SMPI approach includes development of a new class of therapeutic agents with which disease-causing proteins can be destroyed through the SMPI-induced ubiquitination. Current efforts are focused on the development of HIF-1α-octapeptide-replacing non-peptide small molecules.

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20. The incubated cells with SMPI at 37 °C were lysed by lysis buffer (20 mM Tris–HCl pH 7.4, 1% Triton X-100, 5 mM EDTA, 10 μL of Protease inhibitor and 860 μL of distilled water). The protein concentrations were measured using Bio-Rad protein assay reagent. SDS-PAGE was carried out with a 8% SDS separating gel. The separating gel (2.7 mL of 30% Acrylamide/Bis-, 2.5 mL of 1.5 M Tris pH 8.8, 4.6 mL of D.D.W., 50 μL of 20% SDS, 100 μL of 10% APS and 6 μL of TEMED) and stacking gel (1.7 mL of 30% Acrylamide/Bis-, 2.5 mL of 0.5 M Tris pH 6.8, 5.6 mL of distilled water, 50 μL of 20% SDS, 100 μL of 10% APS and 10 μL of TEMED) were prepared just before running. 20 mg of protein samples were loaded from each time points and control. The electrophoresis was performed at 120 V for 2 h using a running buffer system (3.03 g of Tris–Cl, 14.41 g of Glycin, 5 mL of 20% SDS, and distilled water up to 1 L). The proteins were then transferred to PVDF membrane (Amersham) at 250 mA electric current for 2 h using a transfer buffer system (3.78 g of Tris–Cl, 18 g of Clycine, 1.85 mL 20% SDS, and distilled water up to 1 L). The membranes were washed three times with PBST buffer, were then treated with 5% skim milk for 3 h at room temperature (or overnight at 4 °C). After 2 h-incubation with the primary MetAP-2 antibody (1:250 in BSA, Zymed) at room temperature (or overnight at 4°C), the membrane was incubated with the secondary antibody (1:10,000 in 3% skim milk, Amersham) at rt for 2 h. The membranes were then washed with PBST three times. Finally, the film was developed by Western blotting detection reagents (Amersham) using a Kodak film (Kodak X-OMAT AR).

Targeted Degradation of Proteins by Small Molecules: A Novel Tool for Functional Proteomics†

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Abstract: A novel strategy that targets protein for degradation has recently been developed by exploiting a protein-targeting chimeric molecule ('Protac'). Typically, the chimeric Protac is composed of a small-molecule ligand ('bait') on one end and a synthetic octapeptide on the other. This octapeptide is recognized by E3 ubiquitin ligase pVHL (von Hippel Lindau tumor suppressor protein), thereby recruiting a small molecule-bound protein ('prey') to pVHL for ubiquitination and degradation. Since selective degradation of a cellular protein generates a "loss of function" mutation, this protein knock-out strategy may be useful to study the function of a given protein or to evaluate whether a cellular protein is a potential target for drug intervention, in a manner reminiscent of gene knock-out or siRNA approaches. Herein, we show that a synthetic pentapeptide is sufficient to interact with pVHL E3 ligase, and that the pentapeptide-based Protac efficiently induces ubiquitination and degradation of target protein. Our results also demonstrate that the pentapeptide-based Protac can enter cells efficiently to exerts its biological activity effectively. These results suggest that the synthetic pentapeptide can be used either directly in the preparation of cell-permeable Protacs or as a template to develop peptidomimetic or non-peptide Protacs.

Keywords: Protein-targeting chimeric molecule (Protac), ubiquitin, E3 ubiquitin ligase, post-genomic era, hypoxia-inducing factor-1α (HIF-1α), von Hippel Lindau tumor suppressor protein (pVHL), proteasome, estrogen receptor (ER), estradiol (E2).

†This Paper is Dedicated to Professor Dong H. Kim on his 70th Birthday

INTRODUCTION

With the completion of the human genome project, more than 10, 000 genes where the functions of their gene products remain unknown are now available for study. Assigning functions to these proteins is a daunting task in the post-genomic era. There is therefore an urgent needs for additional new strategies to study protein function. Over the years, functional studies of proteins have been carried out by a variety of methods such as gene-knockout, small-molecule modulation (*i.e.* chemical inhibition or activation), antisense, protein overexpression, and more recently small interfering RNAs (siRNAs). While these approaches have greatly contributed in exploring functions of proteins, each has some inherent limitations for large scale protein functional study [1, 2]. For example, while the gene knock-out strategy has proven to be a powerful tool for the study of protein function for many years, it is unsuited for proteins where loss may prove lethal during the development process. In addition, the analyses of knockouts can be complicated due to the irreversible nature of gene manipulation. Most of all, due to the time-consuming nature of this technique, it is not suited for the large-scale functional analyses required for the post-genomic era.

The protein over-expression strategy has also been widely used to study protein function. However, the direct over-expression of dominant-negative proteins may irreversibly alter the intracellular environment and thus it may be difficult to explore their functions precisely without proper regulatory tools. Furthermore, if the over-expressed protein has additional functions or if it interacts with an endogenous counterpart, such strategies can yield misleading results.

Many strategies for ligand-inducible regulation of protein activity have been developed [1, 3], but these approaches are often limited to specific signaling molecules. For example, several systems that are controlled by a small-molecule drug such as tetracycline or rapamycin have been developed to regulate transgene expression pharmacologically at the level of transcription [4-7]. Unfortunately, these systems are appropriate only for proteins that require prolonged kinetics due to a long lag-time between induction and decay of protein expression, preventing precise control of rapid response and cessation. Recently, siRNA, which knocks down the expression of a gene of interest by destroying its mRNA, has also drawn considerable attention as an effective molecular probe as well as a potential therapeutic approach [2, 8], despite some inherent limitations [2, 8, 9], such as off-target and sequence-independent effects. However, the use of siRNAs in a large scale protein functional study has not been explored at this time. In the antisense technology, oligonucleotides or their analogues complementary to specific sequences target mRNA or pre-mRNA, interfering with the expression of target proteins. The antisense technology shares similar characteristics to that of siRNA.

Biologically active small molecules have recently been increasingly used as molecular probes to explore intracellular processes, opening a new avenue of cell biology, namely 'chemical genetics' [10-12]. While many of these biologically active molecules inhibit functions of target proteins and are useful as molecular probes, the systematic development of these bioactive small-molecule modulators is a challenging task, particularly, for a certain protein target

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that is not an enzyme or a receptor; the design of inhibitor for enzyme or receptor is frequently facilitated by the presenece of defined active or ligand-binding sites. Unlike gene knockout and siRNA that result in a "knockout" or "knockdown" of protein levels at the DNA or RNA level, respectively, most small molecules currently available inhibit/activate the protein function at the post-translational level. However, targeted destruction (rather than inhibition) of protein may provide a more effective way to accomplish the removal of a desired gene product than simple inhibition of target protein at the post-translational level. To date, however, there have been few attempts to design small molecules that induce the degradation (rather than inhibition) of a targeted protein in an otherwise healthy cell, thereby achieving the same purpose as gene knockout or siRNA strategies at the post-translational level.

The ubiquitin-proteasome pathway is the principal conduit for protein turnover in all eukaryotic cells. The ubiquitin-dependent proteolysis involves the assembly of an ubiquitin chain on a substrate, which targets the attached protein for degradation by the 26S proteasome [13]. Ubiquitin, a polypeptide of 76 amino acids, is first activated at its C-terminus by adenylation and formation of a thioester bond with the ubiquitin-activating enzyme, E1. Activated ubiquitin is subsequently transferred from E1 to a cysteine residue of a member of the family of ubiquitin-conjugating (E2) enzymes. Finally, ubiquitin is transferred from the E2 enzyme to a lysine residue of a target protein, either directly or with the assistance of a ubiquitin ligase (E3). Although E2s can directly transfer ubiquitin to protein substrates *in vitro*, most physiological ubiquitination reactions are thought to require the participation of an E3. E3s appear to be the primary source of substrate specificity in the ubiquitination cascade, as they have been shown to bind directly and specifically to substrate [14].

Employing this selective E3 ubiquitin ligase:substrate interaction, Deshaies and collaborators sought to artificially target a protein to the SCF (Skp1-Cullin-F-box) complex, the most characterized E3 ligase complex responsible for the ubiquitination of many regulatory proteins such as IκBα protein. To this end, they successfully designed Proteolysis Targeting Chimeric molecules ('Protacs'), using chimeric molecules containing IκBα-derived phosphopeptide (IPP) on one end that binds β-TRCP E3 ligase, a component of SCF E3 ligase complex, and a series of small molecule ligands that selectively bind to target protein. These Protacs have been shown to recruit target proteins MetAP-2, estrogen receptor (ER) and androgen receptor (AR) to the SCF E3 ligase complex for degradation *in vitro* [15, 16]. Unfortunately, the Protac approach had one major drawback;

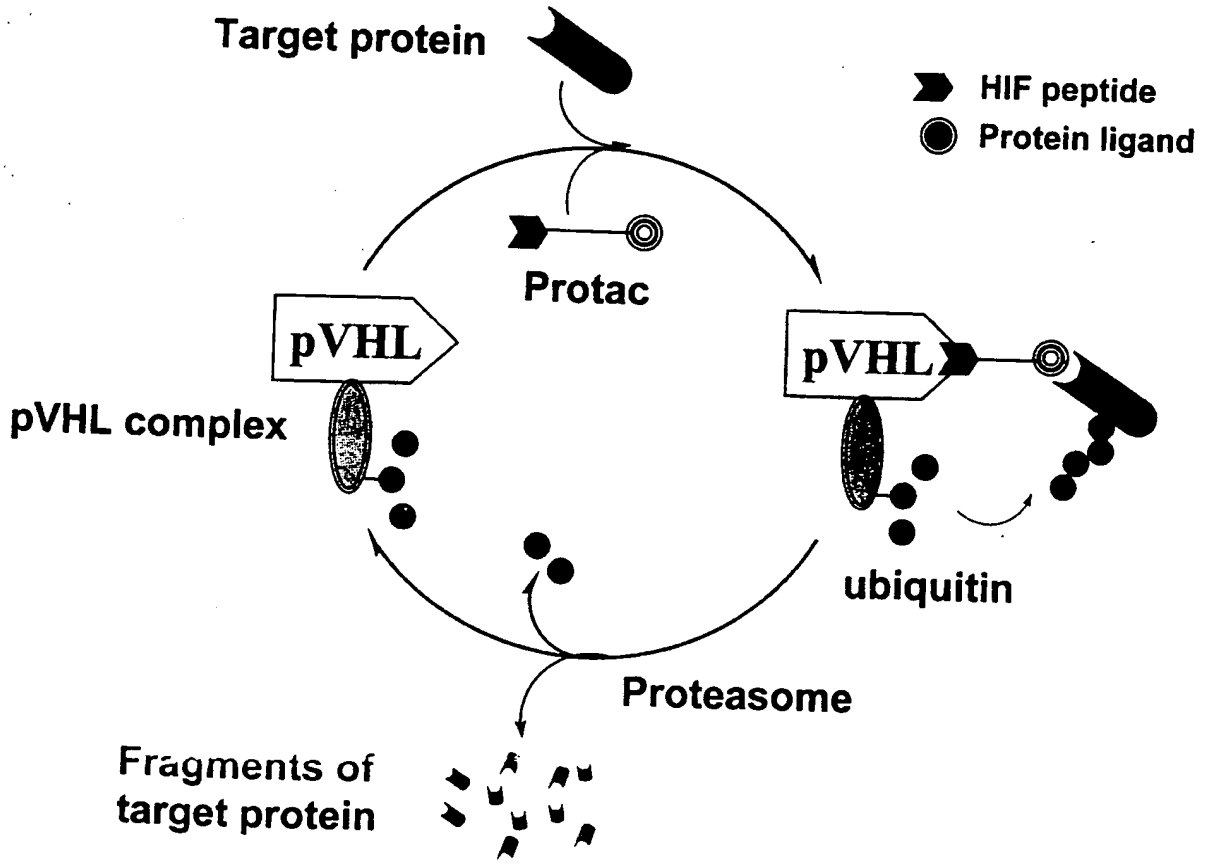


Fig. (1). A cell-permeable protein-targeting chimera (Protac) recruits a target protein to pVHL (von Hippel Lindau tumor suppressor protein) E3 ubiquitin ligase for ubquitination and degradation.

these small molecules lack cell permeability and therefore need to be microinjected into cells. Given the important breakthrough of the Protac technology in effecting specific protein degradation via the ubiquitin-mediated proteasome pathway, we have sought an alternative chemical genetic means to overcome the lack of cell permeability of the first generation of Protacs. Thus, the use of an alternative E3 ligase:substrate interaction was considered; the hypoxia-inducing factor (HIF)-1α has features relevant to this application.

Under normoxic conditions, the hypoxia-inducing factor-1α (HIF-1α) is rapidly degraded by the ubiquitin-proteasome pathway [17]. The degradation of HIF-1α is triggered by the hydroxylation of a conserved proline residue (Pro^{564}) that is subsequently recognized by the pVHL (von Hippel Lindau tumor suppressor) E3 ligase, a component of the ubiquitin ligase complex that targets HIF-1α for degradation by the 26S proteasome [18, 19]. Taking advantage of this pVHL:HIF-1α interaction, we have succeeded in artificially recruiting a target protein to the pVHL for ubiquitination and degradation in living cells, using a cell-permeable protein-targeting chimeric molecule (Protac) [20] (Fig. 1). Typically, a pVHL ligand-based Protac is composed of a small-molecule ligand ('bait') on one end and a synthetic octapeptide (derived from HIF-1α residues 561 to 568) on the other that is recognized by the E3 ubiquitin ligase pVHL, thereby recruiting a small molecule-bound target protein ('prey') to the pVHL for ubiquitination and degradation. In this respect, Crews and colleagues also reported the development of the pVHL-based Protacs that target green fluorescent protein fused FK506 binding protein (FKBP12) and androgen receptor (AR) [21], where they introduced a carrier motif (octa-arginine) to facilitate the membrane transport of Protacs.

Unlike traditional small-molecule approaches that use inhibitors or activators to perturb protein function, the advantage of the Protac approach is that any small-molecule ligands, such as inhibitors, activators or ligands with no biological function, can be used to target proteins for degradation. This potentially leads us to target proteins that are not enzymes or receptors for which designing small-molecule modulators is often a difficult task due to the lack of defined active sites. In addition, Protacs could in principle be used to target almost any protein within a cell and selectively initiate its degradation, resulting in a 'chemical knockout' of protein function. Thus, we envision that the Protac approach may provide a generic tool for scientists to knock down cellular proteins in order to study their physiological functions and identify the pathways in which they are associated, thereby evaluating whether a cellular protein is a useful target for drug intervention as well. However, given the peptidic nature of the pVHL ligand (i.e. synthetic octapeptide) used in the Protac technology it will be important to replace it with a non-peptide or peptidomimetic residue that can be used for not only the protein functional study but also therapeutic interventions (Fig. 2).

In this report, we sought to identify the minimum chain length of HIF-1α peptide residue that would still be recognized by the pVHL. Hopefully, the shortened peptide could be used as a template to develop a peptidomimetic pVHL ligand. Herein, we show that a synthetic pentapeptide-based Protac is sufficient to recruit a target protein to the pVHL for ubiquitination and degradation. Our results also demonstrate that the pentapeptide-based Protac enters cells efficiently and exerts its biological activity effectively. These results suggest that the pentapeptide could be used either directly in the Protac approach or as a template to develop peptidomimetic or non-peptide Protacs with which to exploit in the systematic functional study of proteins.

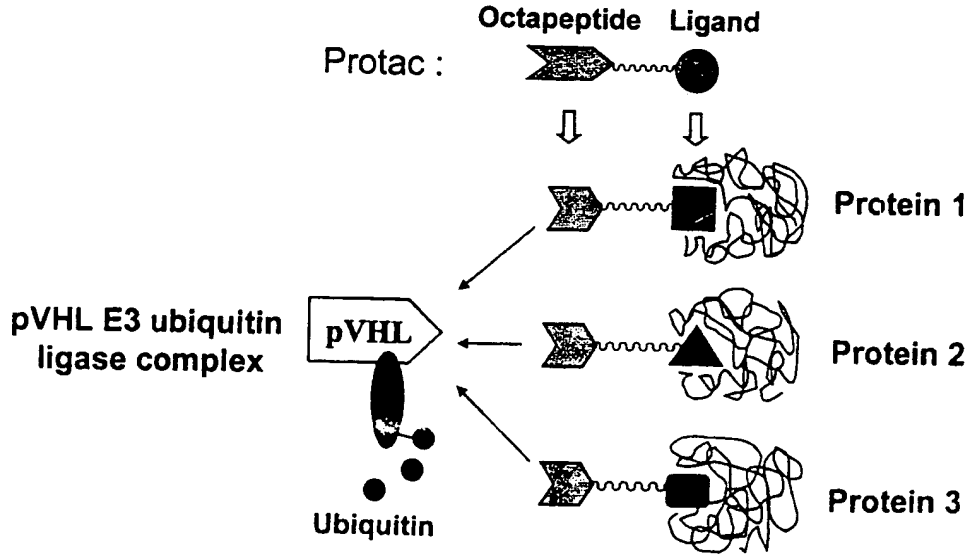


Fig. (2). A schematic of how different proteins might be recruited to pVHL ubiquitin ligase by optimized Protac for ubiquitination and degradation. Protac is composed of a small-molecule ligand on one end and pVHL ligand on the other end.

MATERIALS AND METHODS

Reagents

Fetal bovine serum (FBS), RPMI1640, antibiotics and trypsin-EDTA were purchased from Gibco Co. (Carlsbad, CA). Triton X-100, PMSF, Protease inhibitors cocktail and 2X Laemmi sample buffer were purchased from Sigma Co. (St. Louis, MO). Charcol-dextran treated FBS was purchased from Hyclone Co. (Logan, UT). Cell Proliferation Assay kit was purchased from Promega (Madison, WI). Anti-ERα and anti-ubiquitin were purchased from SantaCruz (Santacruz, CA). Anti-beta actin was purchased from Novus Biologicals Inc. (Littleton, CO). Anti-rabbit IgG was purchased from Zymed Laboratories (South SanFrancisco, CA). Protein assay dye, SDS and PVDF membrane were purchased from Bio-Rad (Hercules, CA). Secondary anti-mouse or anti-rabbit peroxidase-conjugated antibody and ECL enhanced chemiluminescence detection reagents were purchased from Amersham Corp. (Buckinghamshire, United Kingdom). All the organic reagents were purchased from Aldrich Chemical Co.

Synthesis of cell-permeable-Protacs

All of the Protacs described here are prepared following a similar procedure to that previously reported [15, 16, 21]. The final product was characterized by electrospray (ES) mass spectrometry. All other intermediates were characterized by 500-MHz 1H NMR spectroscopy.

Cell Culture

MCF7 human breast cancer cell line was purchased from the American Type Culture Collection (Manassas, VA). MCF7 cells maintained in RPMI 1640 (Gibco, Carlsbad, CA) medium containing 10 % (v/v) fetal bovine serum (Gibco, Carlsbad, CA) and 100 U/mL penicillin-100 ug/mL streptomycin (Gibco, Carlsbad, CA). All experiments were done when the cells were 70 % confluent.

Western Blotting

The sample was mixed with an equal volume of 2 X Laemmi sample buffer (Sigma, St. Louis, MO) and boiled for 10 min. Equal protein concentrations of sample were subjected to 8% SDS polyacrylamide gel, electrophoresed, and blotted onto a PVDF membrane. The membranes were incubated overnight at 4 ^{0}C with rabbit anti-ERα (HC 20) antibody (SantaCruz, Santacruz, CA), anti-ubiquitin (FL 76) antibody (SantaCruz, Santacruz, CA). Membranes were next incubated with anti-rabbit peroxidase-conjugated antibody for 1 hr at room temperature. Protein was detected using ECL enhanced chemiluminescence detection reagents (Amersham Corp., Buckinghamshire, United Kingdom). All membranes were then reprobed with mouse anti-beta actin (AC-15) antibody (Novus Biologicals Inc., Littleton, CO) to ensure equal protein loading.

Cell Proliferation Assay

MCF7 cells were plated at a density of 2 X 10^4 cells per 24 well plates in RPMI 1640 with 10% FBS and left overnight. This was followed by three washes of the cultures in hank's buffered salt solution. Cells were exposed for 3 days to 17 beta-estradiol or E2 coupled peptides as indicated in RPMI 1640 with 5 % charcol-dextran treated FBS (Hyclone, Logan, UT). Control cells were exposed to 0.05 % DMSO. Cells were then detached from the plates by trypsin-EDTA treatment and counted in a hemocytometer. The cell proliferation assays were repeated several times with similar results.

Cell Viability Assay by MTT

MCF7 cells were plated at a density of 5 X 10^3 cells per 96 well plates in RPMI 1640 with 10 % FBS and left overnight. This was followed by three washes of the cultures in hank's buffered salt solution. Cells were exposed for 3 days to 17 β-estradiol or E2 coupled peptides as indicated in a phenol- and serum-free RPMI 1640. Control cells were exposed to 0.05 % DMSO. The proliferation rate of the cells was determined at the indicated time points by using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI) according to the supplier's instruction. The absorbance of the formazan product was measured at 570 nm using the FL 600 microplate fluorescence reader (Bio-Tek, Winooski, VT). The cell proliferation assays were repeated several times with similar results.

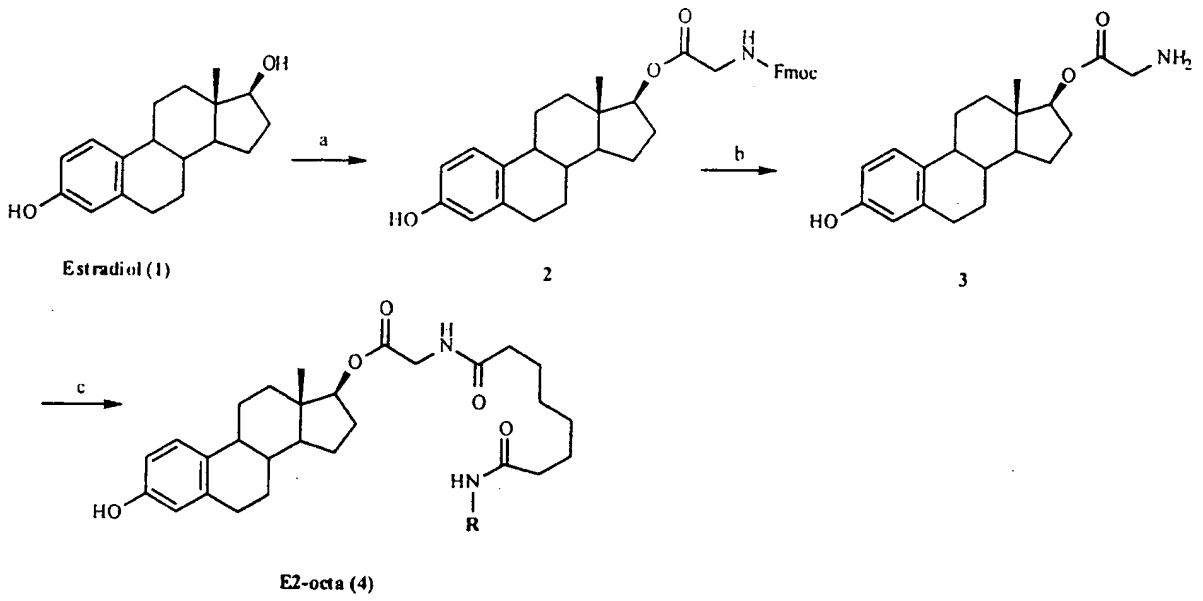
ER Ubiquitination Assays

MCF7 cells were plated at a density of 1 X 10^6 cells per 6 well plates in RPMI 1640 with 10% FBS and left overnight. This was followed by three washes of the cultures in hank's buffered salt solution. Cells were exposed for various time to 17 β-estradiol or E2 coupled peptides as indicated in RPMI 1640 with 5 % charcol-dextran treated FBS (Hyclone, Logan, UT). Control cells were exposed to 0.05 % DMSO. At the end of the incubation, the cells were lysed in cold lysis buffer (20 mM Tris-HCl, pH 7.4; 5 mM EDTA; Protease inhibitors cocktail; 1 % Triton X-100 and 0.2 mM PMSF). The concentration of protein in the cytosol was estimated by the Bradford method (Bio-Rad, Hercules, CA). The cytosol (1mg protein) was incubated with anti-ERα IgG (1:50, SantaCruz, Santacruz, CA) overnight at 4 ^{o}C. To precipitate the antigen-antibody complex, the cytosol was incubated for additional two hours with goat anti-rabbit IgG (1:200, Zymed Laboratories, South San Francisco, CA) at 4 ^{o}C. The immunoprecipitates were pelletd following centrifugation at 10, 000 X g for 15 minutes. The precipitates were then resuspended, electrophoresised, and transferred to PVDF membrane for immunoblotting with anti-Ub antibody.

RESULTS

Design of Cell-Permeable Protacs

To employ the Protac technology for protein functional studies in the post-genomic era (Fig. 2), we first sought to identify the minimum length of HIF peptide that can be used as a template to develop peptide-mimetic or non-peptide Protac. To screen the minimum size of peptide that is still recognized by pVHL, we decided to use estrogen receptor-α (ER) degradation assay. To do this, we first



Scheme 1. Synthesis of estrogen receptor (ER)-targeting Protacs, E2-octa. a. Fmoc-Gly-OH, oxalyl chloride, b. Tetrabutyl ammoniumfluoride (TBAF), c. 1) Disuccimidyl suberate (DSS), 2) HIF-1α octapeptide, R = -Met-Leu-Ala-Pro^{OH}-Tyr-Ile-Pro-Met.

prepared ER-targeting Protacs containing a series of HIF-1α peptides (Scheme **1**). While keeping hydroxyproline in the middle, we deleted amino acids from both ends of HIF-1α octapeptide to prepare a series of shortened peptides. These peptides were then coupled to estradiol to give ER-targeting Protacs following a similar strategy to that reported previously [21]. To evaluate whether shortened peptides interact with pVHL, an ER degradation assay in MCF-7 cells was initially used.

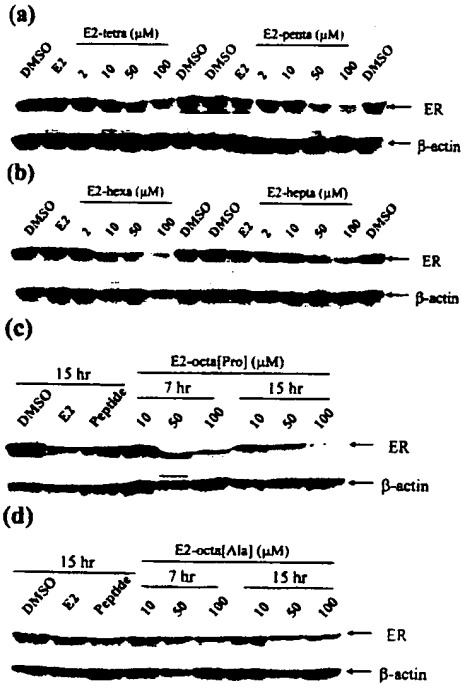


Fig. (3). Western blots of lysates from Protac-treated MCF-7 cells probed with anti-ER antibody, Cells were treated with 2-100 μM of (a) E2-tetra or E2-penta, (b) E2-hexa or E2-hepta, and incubated for 24 hr before cell lysis and immunoblotting. (c) Cells were incubated with 10 μM of E2-penta or E2-octa over 16 hr time period, and lysed and immunoblotted with anti-ER antibody. Cells were treated with 10-100 μM of (d) E2-octa[Pro] or (e) E2-octa[Ala], and incubated for 7 or 15 hr. Cells then were lysed, and immunoblotted with anti-ER antibody.

Protac-Induced ER Degradation in Living Cells

Firstly, MCF-7 breast cancer cells were treated with Protacs for 24 hr, harvested and then the protein level of ER was probed by immunoblotting with anti-ER antibody. As shown in (Figs. 3a and 3b), all Protacs except E2-tetra efficiently recruited ER to pVHL, resulting in ER degradation. E2-penta showed similar degradation efficiency to that of E2-hepta or E2-hexa. When E2-octa[Pro] was treated in MCF-7 cells, it also induced ER degradation (Fig. 3c). Since hydroxyproline is strictly required to interact with pVHL [18, 19], we reasoned that the proline residue of E2-octa[Pro] is first hydroxylated by a HIF prolyl hydroxylase, and then recognized by pVHL for ER degradation. Previously, it was generally thought that HIF prolyl hydroxylase recognizes peptide substrates longer than 20-mer [18, 19], whereas our results with E2-octa[Pro] demonstrated that octapeptide is sufficient to be a substrate for HIF-1α prolyl hydroxylase. Meanwhile, a mutant Protac (E2-octa[Ala]) did not induce ER degradation (Fig. 3d), indicating that interaction between pVHL and hydroproline of HIF-1α peptide is critical for ER degradation. Taken together, our results clearly show that the pentapeptide is sufficient to recruit ER to pVHL for degradation. Therefore, we wanted to further verify whether E2-penta exerts the same biological activity as E2-octa by comparing its ability to ubiquitinate ER and inhibit growth of tumor cells with that of E2-octa.

pVHL-Mediated ER Ubiquitination and Degradation by E2-Penta

To confirm whether E2-penta mediates ER degradation through the ubiquitin-proteasome pathway, we tested whether ER is ubiquitinated in an E2-penta dependent manner, and also whether the ER ubiquitination is mediated by the pVHL E3 ubiquitin ligase. First, we performed an immunoprecipitation experiment with anti-ER-α antibody followed by western blotting for ubiquitin. As shown in (Fig. 4), ER was ubiquitinated in an E2-penta dependent manner at a concentration of 1 μM E2-penta, whereas E2-octa was used as a positive control. It should be noted that E2 is previously shown to induce the ubiquitination of ER [22-24] and thus used as another positive control. Typically, E2 binds ER and recruits co-activators, promoting expression of estrogen-responsive genes [25]. It is thought that coactivator binding to ER is important for E2-mediated degradation of ER [25, 26]. To evaluate whether ER is ubiquitinated by the pVHL E3 ligase complex, we also prepared a mutant E2-penta in which hydroxyl proline is replaced with alanine, and thus the mutant E2-penta[Ala] no longer binds the pVHL E3 ubiquitin ligase. As shown in (Fig. 4), E2-penta[Ala] did not induce ER ubiquitination, implicating that interaction between pentapeptide and pVHL is critical for ER ubiquitination. Taken together, these results confirm that E2-penta induces ER degradation through the pVHL-proteasome pathway, analogous to that of E2-octa.

E2-Penta Blocks Proliferation of Breast Cancer Cells

To determine whether E2-penta has a similar effect to that of E2-octa in the growth inhibition of breast cancer cells, MCF-7 cells were treated with E2-penta and cell numbers were counted in every 24 hr over 72 hr period. Tamoxifen, ICI 182, 780 and E2-octa were used as control, since they inhibit growth of tumor cells. After 48 hr

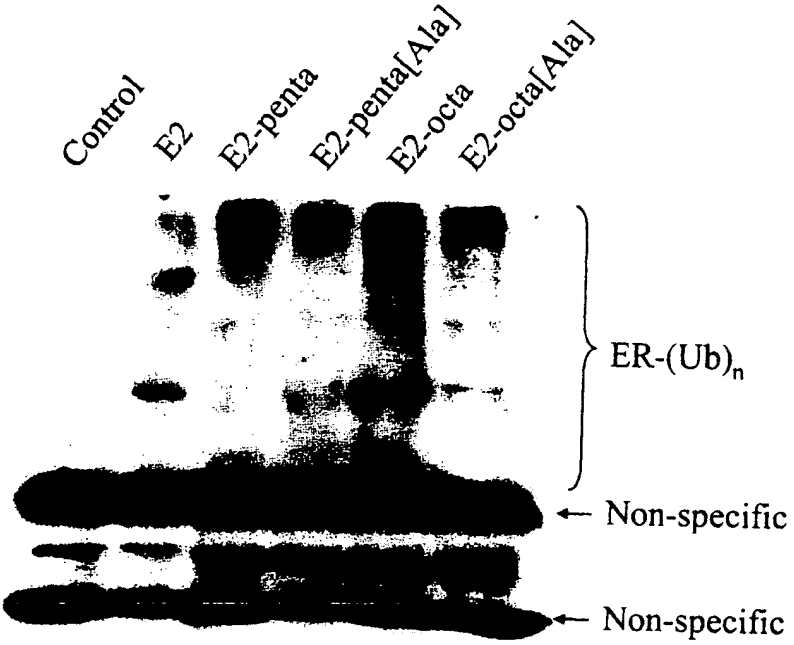


Fig. (4). pVHL-dependent ER ubiquitination by E2-penta. MCF-7 breast cancer cells were treated with 1 μM of E2-penta, E2-penta[Ala], E2-octa, or E2-octa[Ala]. After 4 hr incubation, cells were lysed and incubated with anti-ER antibody and goat anti-rabbit IgG, then immunoblotted with anti-Ub antibody.

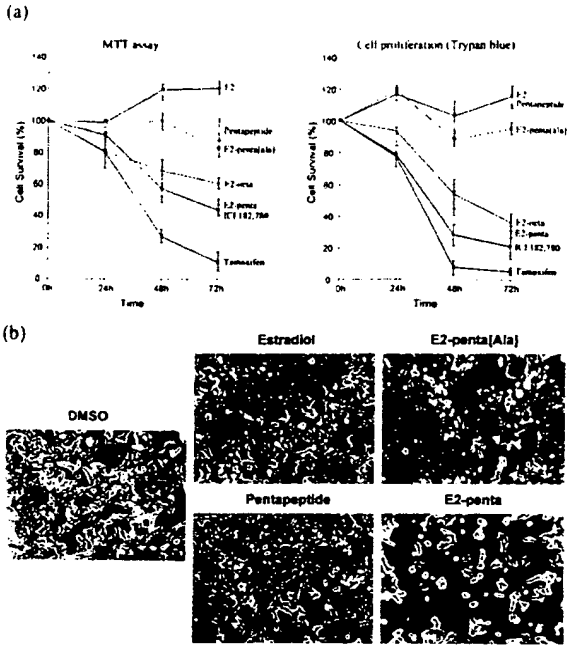


Fig. (5). E2-penta blocks proliferation of MCF-7 cells. (a) Cells were treated with E2 (0.01 μM), E2-penta (50 μM), E2-penta[Ala] (50 μM), pentapeptide (50 μM), tamoxifen (5 μM), or ICI 182,780 (20 μM), and cell survival was measured every 24 hr over 3 days by cell viability assay by MTT assay and cell proliferation assay (Trypan blue stain). All points were done at least in quadruplicate. (b) MCF-7 cells treated with E2-penta. MCF-7 cells were treated with DMSO, estradiol (0.01 μM), pentapeptide (50 μM), E2-penta (50 μM), or E2-penta[Ala] (50 μM) for 48 hr. Magnification is X20.

incubation with E2-penta, cell numbers dramatically decreased (Fig. 5a), whereas E2-penta[Ala] did not show the same effects as E2-penta. These results suggest that E2-penta-induced ER degradation is closely correlated with the growth inhibition of MCF-7 cells. Meanwhile, (Fig. 5b) shows cells treated with 50 μM of E2-penta or mutant E2-penta after 24 hr. The majority of cells treated with E2-penta ceased growing and died after 48 hr. Taken together, E2-penta appears to be more effective in the growth inhibition of MCF-7 tumor cells than E2-octa.

Inhibition of ER-Regulated Protein Expression by E2-Penta

Based on the important role of ER-promoted gene expression in tumor cell growth [27], it is likely that ER degradation and subsequent growth inhibition of tumor cells by E2-penta are a consequence of Protac's ability to inhibit the estradiol-stimulated expression of E2-responsive-genes that promote cell proliferation. Thus, we wanted to determine whether E2-penta indeed inhibits the expression of ER-controlled gene products. For this study, MCF-7 breast cancer cells were pretreated with E2-penta or E2-penta[Ala] for 2 hr, and E2 in fresh medium was then added to promote expression of ER-controlled genes. After additional 2 or 4 hr incubation, the protein level of progesterone receptor (PR) whose gene expression is normally promoted by the estrogenic action was probed by immunoblotting with anti-PR antibody. As shown in (Fig. 6), after 2 hr incubation with E2, PR protein level was not up-regulated, probably due to the ER degradation by E2-penta. However, after 4 hr incubation, its ability to degrade ER was diminished that the level of PR protein was increased. We believe that E2-penta become ineffective in living cells within 4 hr of incubation at that concentration. It may be possible that the pentapeptide motif of E2-penta is hydrolyzed by peptidases present abundantly in living cells, thereby losing its ER degradation activity. Alternatively, cells may produce ERs constantly, which promotes E2-stimulated estrogenic action. Therefore, it is important to develop a non-peptidic or peptide-mimetic pVHL ligand that may be used for the preparation of stable, catalytic Protacs. Taken all together, our results indicate that E2-penta inhibits proliferation of breast cancer cells in a similar extent to E2-octa, suggesting that the pentapeptide motif may be useful as a template in the development of non-peptidic or peptide-mimetic Protacs, replacing HIF-1α octapetide motif.

DISCUSSION

Since the completion of the human genome project, it is clear that there is an urgent need for a more systematic approach to studies on protein function. Major concerns over current strategies, such as gene knockouts, relate to a lack of generality and timeliness and to complexity of analyses. Recently, Deshaies and colleagues developed Protac technology, an approach that targets a protein for degradation via the ubiquitin-proteasome pathway *in vitro*. We have now developed a cell-permeable Protac that targets the nuclear receptor ER [20]. The Protac approach exploits characteristics of the eukaryotic intracellular degradation system, the ubiquitin-proteasome pathway. Unlike genetic

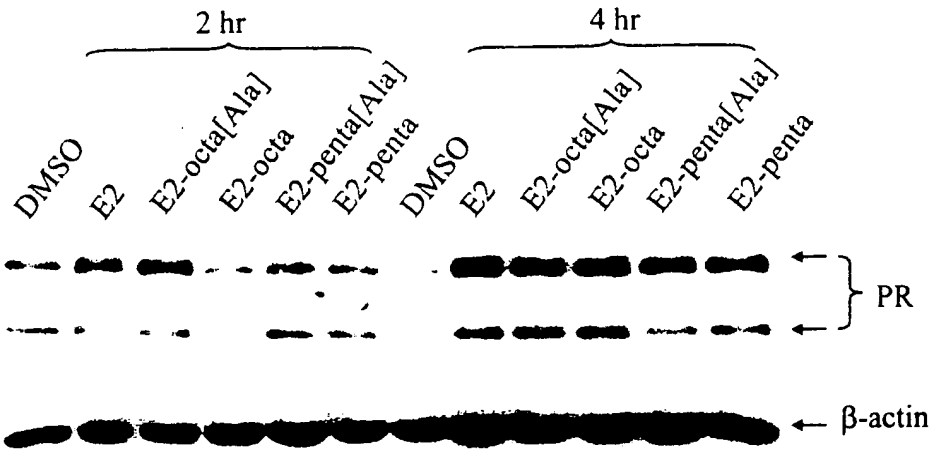


Fig. (6). E2-penta inhibits expression of progesterone receptor (PR). MCF-7 cells were treated with 1 μM of E2-octa, E2-octa[Ala], E2-penta, or E2-penta[Ala] for 2 hr, and subsequently E2 (0.1 μM) was treated with fresh medium for 2 or 4 hr. Cells were then lysed, and immunoblotted with anti-PR antibody.

approaches which knockout gene products of interest at the DNA or RNA level, the Protac approach provides a protein knock-down system at the post-trnaslational level that can be easily turned on and off. This potentially provides a means to carefully analyze the loss of protein function at any stage of cell developmental process. This approach is potentially applicable to the systematic functional study of proteins, provided that small-molecule ligands are available for proteins of interest. Fortunately, there have been considerable efforts toward developing or screening small-molecule ligands [28]. The phage display strategy, for example, which is used to screen peptide ligands for target protein [29], may provide a powerful tool for the broader application of the Protac strategy in chemical genetics. Replacing the octapeptide handle with non-peptide or peptide mimic motif also represents another important step towards the use of Protac approach in functional proteomics. In this report, we successfully sought to screen the minimum size of octapeptide-replacing peptide that can be used in the preparation of Protac and that isstill recognized by the pVHL. Ultimately, this optimized peptide will be used as a template to develop non-peptide or peptidomimetic residues. In this report, we have also shown that pentapeptide-based Protac efficiently induces ubiquitination and degradation of target protein (ER). Our results also clearly show that the pentapeptide-based Protac is as equally efficient as the octapeptide-based Protac in the growth inhibition of breast cancer cells.

Recently, siRNAs have drawn considerable attention as a novel tool to study protein function as well as a potential therapeutic strategy [8] by knock-down of proteins at the

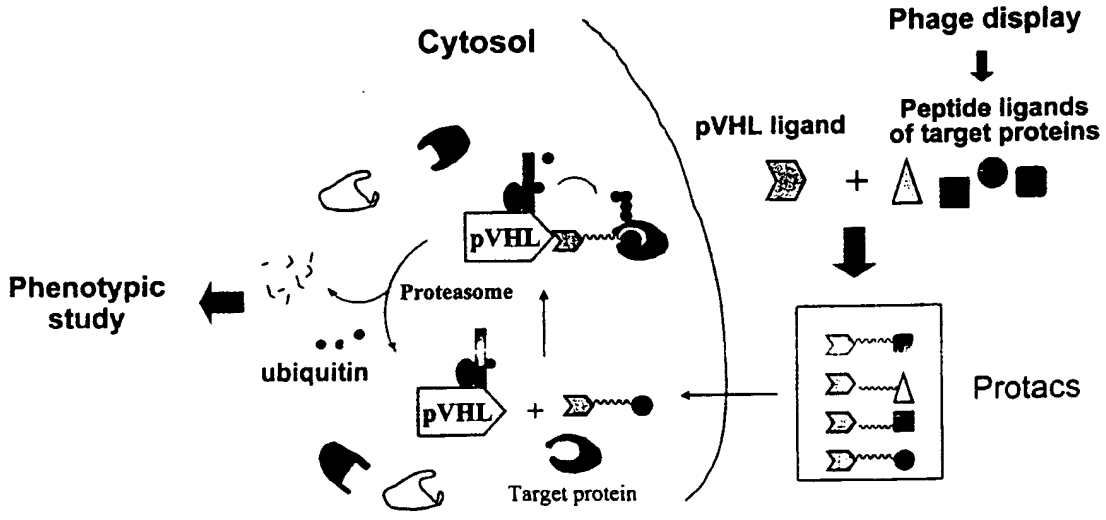


Fig. (7). Chemical genetic approach by Protacs to study functions of proteins uncovered by the Human Genome Project.

RNA level. On the other hand, Protac removes protein of interest at the post-translational level. The Protac-initiated protein degradation could be precisely controlled for accurate biological analysis, since reversible small molecules are easily removable. Another advantage of the Protac approach is that any small molecule ligands (i.e. functional or non-functional ligands are normally of no use either as a molecular probe or as a potential therapeutic agent. The phage display approach could play an important role in the general application of Protac technology to study protein function in the post-genomic era. Phage display technology has proven that it is a powerful tool for screening peptide ligands for protein of interest [29]. Both non-functional and pharmacologically active peptides screened from phage display technique will be useful for the design of Protacs (Fig. 7).

In summary, we have shown that a synthetic pentapeptide-based Protac (E2-penta) is sufficient to induce ubiquitination and degradation of ER, and that E2-penta efficiently enters cultured cells and exerts biological activity. These results suggest that the pentapeptide can be used either directly in the preparation of cell-permeable Protac, replacing the octapeptide motif, or as a template to develop peptidomimetic or non-peptide HIF-1α peptide-replacing small molecules. Development of non-peptidic or pentapeptide-mimetic pVHL ligands could facilitate the use of Protac for large-scale functional study of proteins available in the post-genomic era. At the same time, Protac approach provides an efficient means to evaluate whether target proteins play an important role in disease development and progression, and can target proteins for degradation that are not enzymes or receptors and thus are not typically considered as 'drugable targets'.

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